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Molecular genetic studies of DNA: DNA relatedness and symbiotic genes of Rhizobium japonicum

Robert Vernon Masterson
Iowa State University

Masterson, Robert Vernon

MOLECULAR GENETIC STUDIES OF DNA:DNA RELATEDNESS AND SYMBIOTIC GENES OF RHIZOBIUM JAPONICUM

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Molecular genetic studies of DNA:DNA relatedness and symbiotic genes of *Rhizobium japonicum*

by

Robert Vernon Masterson

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I. INTRODUCTION

A. Genetics of *Rhizobia*

Rhizobia are gram-negative bacteria capable of symbiosis with legume plants. Each genus of *Rhizobia* specifically recognizes the root cells of its host legume. The symbiosis involves a complex series of events, all of which are not understood at this time, and occur such that the bacteria invade, cause plant cell division, and aid in the formation of a root nodule. The nodule is a structure directly attached to a root and contains *Rhizobia* in a morphologically altered, bacteroid form. It is the bacteroids that reduce atmospheric nitrogen to ammonia for assimilation by the plant. In return, the plant supplies the bacteria with nutrients and an ecological niche.

The ability to fix nitrogen is limited to approximately 19 bacterial strains, of which eight of these are *Rhizobia*. The *Rhizobia* have been divided into two groups based on their doubling times. The slow-growing types include *R. japonicum*, *R. lupini*, and a group termed 'Cowpea' species. The doubling times vary greatly but are generally between 6 to 13 h (Denarie et al., 1981). The fast-growing *Rhizobia* include *R. meliloti*, *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*; doubling times are between 2 and 4 h. Recently, new strains of

fast-growing Rhizobium were discovered in the People's Republic of China. These fast-growing strains were found in root nodules of Chinese soybean cultivars, and hence were termed fast-growing R. japonicum (Keyser et al. 1982). Other slow- and fast-growing Rhizobium strains have been identified and include R. loti and members of the Rhizobium 'cowpea' species (Brockwell, 1980).

Although the Rhizobia are gram-negative bacteria, the genetic methods used are generally not the same as those for E. coli, the most well characterized, gram-negative bacterium. There are several reasons for this. First, the properties of nitrogen fixation and nodulation are not readily scorable phenotypes, like auxotrophic markers, and must therefore be tested in planta. Mutagenesis experiments for specific mutants of these traits may require thousands of plants (Long et al., 1982). Slow-growing R. japonicum may be tested in the free-living state for nitrogen fixation, although selection of mutants which do not reduce atmospheric nitrogen still requires examination of formitable numbers of single isolates. Another problem encountered in the study of Rhizobia genetics is lack of transduction, transformation, and congugation methods which are so effective in other systems, such as E. coli. Modifications of existing methods, combined with recombinant DNA technology, has led to new advances in Rhizobia genetics. Recently, Martin and Long (1984) developed a system of generalized transduction in R. meliloti. This is, however, the first and only such system reported in the Rhizobia. Transformation

systems have generally not been developed (Denarie et al., 1981). Conjugation in Rhizobia, involving the horizonal transfer of plasmids between strains, has been effectively used. Examples exist in nearly all strains of Rhizobia in which plasmid-borne sequences are transferred to new genetic backgrounds for examination (Brewin et al., 1980; Hombrecher et al., 1981; and Johnston et al., 1978). In these cases, large endogenous plasmids, harboring nodulation and nitrogen fixation genes, were transferred within and between Rhizobia species. A conjugation method effective in E. coli for mapping large segments of the chromosome using mobilizing plasmids, are now working in various Rhizobia strains. For instance, R-prime plasmids have been shown to mobilize 100-200 Kb regions of a large plasmid in R. meliloti (Banfalvi et al., 1983). The large DNA regions of the R. meliloti genome were moved into a recipient E. coli strain where genetic manipulations were easier to perform.

Another aspect of genetics in an early stage of development in Rhizobia is the ability to directly clone DNA in a Rhizobium background. Virtually all kinds of DNA may be stored and genetically altered in E. coli. This is due to the extensive knowledge of the genetics of E. coli and the developement of strains with properties capable of accepting foreign DNA. In the Rhizobia, host recombination and modification systems are only beginning to be understood such that a cloning system could be established. As stated before, transformation systems are virtually non-existent in the Rhizobia and

must therefore be developed in order to clone and manipulate DNA.

An important feature common between all the Rhizobia is the presence of large plasmids. This turns out to be an advantage as genes coding for symbiosis are commonly located on the plasmids in most of the strains. Such plasmids are termed Sym plasmids and are capable of conferring new host-range specificity to other Rhizobia species (Hombrecher et al., 1981). The plasmids found in the different Rhizobia species generally range in size from 90 to 200 megadaltons and may be isolated by cesium chloride ethidium bromide bouyant density centrifugation (megadalton, mdal.; 1 mdal is equal to 1.5 Kb). Conjugation of plasmids in Rhizobia has defined Sym plasmids in various species: R. leguminosarum (Hirsch et al., 1980), R. leguminosarum and R. phaseoli (Hombrecher et al., 1981), and R. meliloti (Banfalvi et al., 1981). These methods are, however, very tedious and time consuming. Plant tests are required and relatively few strains can be examined at any given time.

The developement of the Southern blot and hybridization techniques (Southern, 1975) has greatly advanced Rhizobia genetics. Radioactively labeled DNA probes, containing specific sequences such as nitrogen fixation genes, have been effectively used to search for homologous sequences bound to nitrocellulose paper. The location of symbiotic genes was rapidly determined by various groups using hybridization to plasmid DNA separated by agarose gel electrophoresis. In this way, Nuti et al., (1979) showed the presence of structural nif

DH, genes on plasmids of various fast-growing strains. Rosenberg et al., (1982) gave evidence supporting the location of nif genes on a megaplasmid (greater than 300 mdal) in R. meliloti. At the same time, Masterson et al., (1982) showed nif genes were located on the plasmids of fast-growing R. japonicum while the slow-growing R. japonicum harbor nif genes on the chromosome or megaplasmid. The hybridization results in these studies were usually obtained within seven days and did not require plant tests.

The source of the structural nif DH genes used in the above studies was either Klebsiella pneumonia or R. meliloti (Ruvkun and Ausubel, 1980). The hybridizations are based on the observation that the structual nif DH genes are highly conserved in 19 of 19 nitrogen fixing bacterial strains (Ruvkun and Ausubel, 1980). This strong amount of conservation of nif translated genes is unusual and perhaps unique among prokaryotic organisms. Other gene sequences used as hybridization probes are not nearly as conserved as the nif structural genes. Nodulation genes cloned from R. meliloti, using an elaborate complementation method, have provided a molecular probe for examination of homologous sequences in other Rhizobia (Long et al., 1982). Homology of the cloned nod sequence from R. meliloti has been demonstrated, to another fast-growing species, with a known nod gene sequence in R. trifolii (Schofield et al., 1983). The same nod probe shows homology with plasmid DNA from fast-growing R. japonicum under standard hybridization conditions. Only under reduced stringency

conditions did DNA sequences from slow-growing R. japonicum show relatedness (Masterson et al., manuscript submitted for publication).

An important aspect of the genetics of the Rhizobia is physical and genetic maps of symbiotic coding regions on the chromosome or Sym plasmid. While maps of specific nif and nod regions have been presented, few examples are available at this time in the Rhizobia of maps of whole plasmids. Prakash et al., (1982) showed a restriction map and location of nif genes of a 225 kb plasmid from R. leguminosarum. A 225 kb non-symbiotic plasmid from R. meliloti was mapped with restriction enzymes by Banfalvi et al., (1981). The only other study available at this time is a physical and genetic map of a 135 kb region of a megaplasmid, containing symbiotic genes, in R. meliloti (Kondorosi et al., 1984). Physical mapping by restriction enzymes of the large plasmids in Rhizobia present a number of difficulties. Among these are problems in getting sufficient quantities of plasmid DNA for restriction digest analysis and cloning. The recent advent of cosmid cloning, allowing cloned inserts up to 45 kb, are now being used in the Rhizobia as shown in R. meliloti by Long et al., (1982).

B. Dissertation Format

The format chosen for this dissertation is the alternate format. In this section, a general introduction to the genetics of Rhizobia,

and specifically R. japonicum, has been presented. The following three sections concern related aspects that constitute a majority of the work done to satisfy the requirements for a Ph.D. in this department. The first section is about the location of nif genes in slow- and fast-growing R. japonicum. The use of the Southern blot and hybridization methods rapidly showed a distinct difference in the location of nif genes in these strains. The table in the first section concerning the molecular weights of the plasmids in the slow- and fast-growing R. japonicum was done by Paul F. Russell, and may be found in Russell and Atherly (1981). The next section addresses the relatedness of the DNA sequences surrounding the symbiotic genes and plasmid DNA in general. Restriction enzyme digests, usually with EcoRI, of total or plasmid DNA were used to determine the fragment(s) which had homology with various probes. Plasmid DNA from a fast-growing R. japonicum strain was hybridized to EcoRI digested plasmid and total DNA of other fast-growing strains isolated from different locations in the People's Republic of China. The same experiment was done with a plasmid from a slow-growing R. japonicum strain with plasmids and total DNA from other slow-growing strains, also from diverse locations. Also, cross homology between the plasmids of the slow- and fast-growing strains was examined.

The second section also examines the hybridization of nif and nod probes to EcoRI digested plasmid and total DNA of both slow- and fast-growing strains. Conservation of similar sized restriction

fragments which hybridized to the same probe was examined in these strains. R. K. Prakash subcloned a 3.5 kb nod fragment from a R. meliloti cosmid clone and this was used in this section as a molecular probe.

The third section of this dissertation presents evidence for the physical and genetic map of large regions of the Sym plasmid pRjaPRC193. This plasmid is from fast-growing strain PRC193 and is approximately 350 kb in size. Both nod and nif genes have been assigned to specific locations on a large, 240 kb mapped region. Over 90% of the plasmid has been mapped and involved the use of a variety of techniques including cosmid cloning, Southern hybridizations, and a novel technique, Southern cross restriction mapping. The problems associated with mapping a large piece of DNA are included in this section.

The last section is a general discussion of the results shown in this dissertation. The relatedness between the slow- and fast-growing strains is discussed with emphasis on the common symbiosis genes. The significance of various features of the physical and genetic map including large regions of the Sym plasmid pRjaPRC193 is also discussed.

II. LOCATION OF NIF GENES IN RHIZOBIUM JAPONICUM

A. Introduction

The symbiotic bacterium Rhizobium japonicum inhabits and fixes atmospheric nitrogen in the root nodules of soybeans. Rhizobium species include fast- and slow-growing types, of which R. japonicum is a slow-growing species with a doubling time of 6 to 13 h. Recently, however, fast-growing R. japonicum strains (doubling times of 4 to 6 h) from the People's Republic of China have been examined. These strains physiologically resemble fast-growing Rhizobium species and yet still nodulate soybean plants (Keyser et al., 1982). Furthermore, Keyser et al. (1982) found that fast-growing R. japonicum form symbiotic relationships with the soybean cultivar Peking but are generally ineffective with common North American soybean cultivars.

A common feature of both fast- and slow-growing R. japonicum, as well as other Rhizobium species, is the presence of large plasmids (Casse et al., 1979; Denarie et al., 1981; Gross et al., 1979; Hirsch et al., 1980; Nuti et al., 1977; Russell and Atherly, 1981). These plasmids range in size from 90 Mdal (megadaltons) to well over 300 Mdal. Plasmids larger than 300 Mdal are termed megaplasmids and have been identified in different Rhizobium species (Denarie et al., 1981; Rosenberg et al., 1982). The slow-growing R. japonicum strains

examined in this study usually contained one large plasmid ranging between 118 and 196 Mdal. The fast-growing strains contained 1-3 plasmids ranging from 54 to 240 Mdal. Therefore, large plasmids are a consistent feature in both slow- and fast-growing R. japonicum.

The structural nitrogen fixation genes are highly conserved in all nitrogen fixing bacteria which is a unique phenomenon in that most translated genes in bacteria are not highly conserved (Ruvkun and Ausubel, 1980). The structural nif genes of Klebsiella pneumonia, cloned by Cannon et al., (1979), were used by Ruvkun and Ausubel (1980) to determine the conservation of nif genes in nitrogen fixing organisms. Genes responsible for nitrogen fixation have been found, using K. pneumonia or R. meliloti structural nif genes as ^{32}P-labeled probes, on either plasmids (including megaplasmids) or the chromosome of the various Rhizobium species (Hirsch et al., 1980; Hombrecher et al., 1981; Nuti et al., 1979; Prakash et al., 1981; Rosenberg et al., 1982). Therefore, a strong advantage exists in using cloned structural nitrogen fixation genes (nifDH) as a hybridization probe in order to identify nif genes in R. japonicum. This paper reports the location of structural nif genes in relationship to the large plasmids present in fast- and slow-growing R. japonicum.

B. Materials and Methods

1. Bacterial strains and media

R. japonicum strains were obtained from the following sources:

61A76, W. Brill (University of Wisconsin, Madison, WI); 3I1b31, E. Schmidt (University of Minnesota, St. Paul, MN). All other R. japonicum strains listed in Table 1 were obtained from the USDA, Beltsville, MD. D. Berryhill (North Dakota State University, Fargo, ND) provided R. phaseoli strain DB1 and R. meliloti strains 102F28 and 102F51. A. tumefaciens strain A277 was provided by M. D. Chilton (Washington University, St. Louis, MO). E. coli strain HB101, containing plasmid pRmR2 (pACYC184 and nifDH), was obtained from G. Ruvkun and F. Ausubel (Harvard University, Cambridge, MA).

Rhizobium and Agrobacterium cells were grown in TY medium (Berenger, 1974) which contained per liter: 5 g Bacto-tryptone (Difco); 3 g yeast extract; 0.93 g $CaCl \cdot 2H_2O$. E. coli were grown in standard LB medium which contained per liter: 10 g Bacto-Tryptone (Difco); 10.0 g NaCl; and 5 g yeast extract.

2. Plasmid isolations and gel electrophoresis

The plasmid isolation techniques of Casse et al. (1979) and Hirsch et al. (1980) were used in the isolation of large plasmids from Rhizobium, Agrobacterium, and E. coli. The specific details of these isolation procedures are described in Section II. A significant aid in the extraction of intact, covalently closed circular DNA from slow-growing R. japonicum was the addition of a wash step prior to cell lysis. This consisted of resuspending cells in 3 % NaCl for as long as one hour as suggested by Russell and Atherly (1981). Plasmid

DNA was resuspended in low TE (10 mM Tris, pH 8.0; 1 mM EDTA) and kept at 4^{o} C until needed for agarose gel electrophoresis. CsCl-ethidium bromide gradients were not necessary for the separation of the large plasmids from Rhizobium and Agrobacterium, but were required in the extraction of pure pRmR2 used in the ^{32}P-labeling reaction. A tracking dye consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% (w/v) sucrose in H_2O was diluted six-fold with approximately 1 ug of plasmid DNA in 100 ul of low TE. Plastic pipette tips (Denville) were cut off at the tip to produce a larger bore size in order to reduce shear forces on the plasmid DNA when applied to agarose gels.

A vertical gel apparatus was used in the separation of large plasmids. The apparatus contained 0.7% agarose (Seakem) prepared in TBE buffer (0.09 M Tris, pH 8.3; 0.09 M boric acid; and 2.5 mM EDTA). The agarose gel, approximately 130 x 130 x 3 mm and consisting of ten possible slots for DNA, was suspended in TBE buffer prior to the loading of DNA samples. Each sample was carefully layered in the individual slots (also referred to as lanes) and electrophoresis was conducted at 100 V for 5 h at 6 to 8^{o} C. After this time, the gel apparatus was carefully taken apart and the agarose gel was stained in water containing 1 ug/ml ethidium bromide for approximately 15 minutes. The gels were routinely photographed using a Polaroid MP4 apparatus with Polaroid 665 film. A Wratten 23 and a gelatin filter (Kodak) were used to optimize the photographic conditions. Gels suitable for Southern blotting were irradiated with short-wave UV

light for 15 minutes to produce nicks in the large plasmids such that efficient transfer to nitrocellulose would occur.

3. Southern blots and labeling reactions

The hybridization procedure of Southern (1975), modified by Haugland and Verma (1981), was used in the transfer, hybridization, and autoradiography of the plasmids listed in Table 1. Nitrocellulose filters (Millipore) were cut to the same size as the agarose gel and 20X SSC (1X SSC: 0.15 M NaCl; 0.015 M Na-citrate) was used, with paper towels as wicks, in the Southern transfer process. Ten ml of hybridization solution (6X SSC; 1% sarkosyl; 0.5 mg denatured salmon sperm DNA; and 5% dextran sulfate, in water) were added to a plastic pouch containing the nitrocellulose filter and sealed by heat. The bag was placed in a shaking water bath set at 65^{0} C overnight.

Approximately one ug of CsCl-gradient-purified pRmR2 was labled with alpha ^{32}P-dCTP to a specific activity of 1 X 10^{6} to 1 X 10^{7} cpm/ug according to the procedure of Rigby et al. (1977) using a nick translation kit (New England Nuclear). Labled plasmid DNA was separated from unincorporated nucleotides by column chromatography using Sephadex G 50-80 (Pharmacia) in low TE with a sterile, siliconized, 5 ml glass pipette. The radioactive unincorporated nucleotides sifted slowly through the bead matrix while the plasmid DNA, too large for the beads, passed relatively rapidly aroud the beads. A hand held Geiger counter (Lionel) was used to monitor the

progress of the labled plasmid DNA through the Sephadex and 0.4 to 0.6 ml was routinely collected. The labled plasmid DNA was denatured, added to one ml of hybridization solution, and added to the hybridization bag containing the filter which had pre-hybridized overnight. The hybridization was resumed at 65^{o} C for 36 to 40 hours in a shaking water bath. At this time, the hybridization solution was carefully removed and discarded. Labled plasmid DNA which did not hybridize was removed by adding a wash solution (2X SSC; 0.1% sodium dodecyl sulfate) and placed at 65^{o} C while shaking for 20 minutes. This step was repeated once and the filters were washed at room temperature with the wash solution until the background counts decreased significantly. This usually required 4 to 5 changes of the wash solution over a 2 hour period. The hybridization filter was then wrapped in plastic, covered with a sheet of X-ray film (Kodak), and placed in the dark overnight. If further exposure was necessary, an intensifying screen (Dupont) was used and the exposure was repeated.

Colony hybridizations were performed essentially as described by Grunstein and Hogness (1975) using 85 mm circular nitrocellulose membranes (Millipore). Different bacterial colonies were spread over 2 to 4 mm areas on the hybridization filters. The filters were placed on paper towels soaked with denaturation solution (0.5 M NaOH; 1.5 M NaCl) for 10 minutes and then were transferred to paper towels soaked with renaturation solution (0.5 M Tris, pH 8.0; 1.5 M NaCl) and allowed to stand for 15 minutes. Each filter was placed in a suction

apparatus and rinsed once with chloroform and three times with 95% ethanol. A vacuum oven, preset at 80^{0} C, was used in order to adhere single-stranded DNA to the filters. The filters were placed in the oven and allowed to bake over a two hour period. Hybridization conditions were the same as previously described for the Southern hybridization blots.

C. Results and Discussion

1. Nitogen fixation genes in R. japonicum

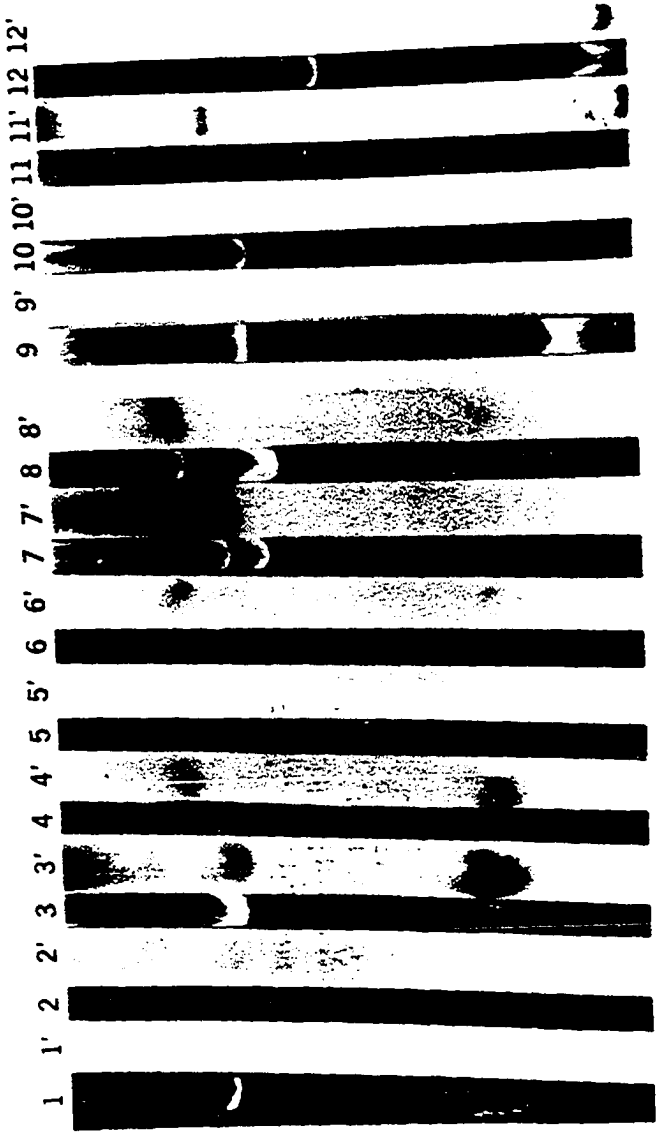
The structural nifKDH genes and part of the nifE gene have been cloned from Klebsiella pneumoniae resulting in the recombinant plasmid pSA30 (Cannon et al., 1979). The nifD and nifH genes code for a subunit of nitrogenase and nitrogenase reductase, respectively, and show a high amount of homology when hybridized to all other nitrogen fixing prokaryotes (Ruvkun and Ausubel, 1980). The recombinant plasmid pSA30 was used by Ruvkun and Ausubel (1980) in order to clone the structural nitrogen fixation genes from R. meliloti. This resulted in the recombinant plasmid pRmR2 which contains a 3.9 Kb EcoRI fragment containing nifDH. Although the amount of nitrogen fixation (nif) relatedness between the nitrogen fixing prokaryotes is remarkably high, the use of nif genes from a Rhizobium species seemed to be a better choice in terms of use as a hybridization probe to the DNA of R. japonicum. For this reason, pRmR2 was labeled with ^{32}P-dCTP by the

nick translation procedure (Rigby et al. 1977) and was subsequently hybridized with the DNA of R. japonicum in order to determine the location of the structural nif genes.

Two different types of hybridizations were performed in order to confirm the location. First, the Southern hybridization procedure (Southern, 1975) was used in the transfer of separated plasmids to nitrocellulose so that a determination of whether or not nif genes were on the large plasmids could be made. Second, colony hybridizations (Grunstein and Hogness, 1975) were performed for the determination of nif hybridization with the total DNA of the slow- and fast-growing R. japonicum. The colony hybridizations were used as a rapid assay in that 2 to 4 mm spreads of bacterial cells on nitrocellulose were sufficient for the detection of nif genes. This method circumvented the isolation and purification of total DNA suitable for hybridizations from each individual strain. Each hybridization procedure used the same hybridization conditions described by Haugland and Verma (1981). The pRmR2 plasmid DNA was labeled in exactly the same manner each time (Rigby et al., 1977) and approximately the same amount of labeled probe was used in each hybridization assay.

The results of the Southern hybridization to the separated large plasmids of the Rhizobium strains examined are shown in Figure 1. The size of the plasmids in Mdal range from 54 to 300 as reported by Russell and Atherly (1981). Determinations of plasmid molecular

Figure 1. Hybridization of ^{32}P-labeled *R. meliloti nif* DNA to plasmids listed in Table 1 Numbers 1-12 (unprimed) are ethidium bromide stained plasmids separarated by agarose gel electrophoresis. Primed numbers are audioradiograph results of the hybridization. Lane 1, *A. tumefaciens* A277; lane 2, *R. meliloti* 102F28; lane 3, *R. meliloti* 102F51; lane 4, *R. phaseoli* DB1; lane 5, *R. japonicum* PRC194; lane 6, *R. japonicum* PRC201; lane 7, *R. japonicum* PRC205; lane 8, *R. japonicum* PRC206; lane 9, *R. japonicum* 61A76; lane 10, *R. japonicum* AA102; lane 11, *R. japonicum* PRC193; and lane 12, *R. japonicum* PRC191.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weights were done by plotting the relative migration of the plasmid DNA through an agarose gel versus the log of a molecular weight standard. A. tumefaciens strain A277 was used by Russell and Atherly (1981) as the molecular weight standard. The plasmid isolation procedures of Hirsch et al. (1980) and Casse et al. (1979) were used in the isolation of plasmids in this study. Plasmids larger than 300 Mdal were not detected using these particular plasmid extraction and electrophoresis conditions. Lanes 1 to 8, as well as 11 and 12, contain plasmid DNA isolated from A. tumefaciens and fast-growing Rhizobium strains. In lanes 9 and 10, plasmid DNA from two slow-growing R. japonicum are represented. Next to each individual lane is the Southern hybridization result; numbers which are primed in Figure 1 are represented with the symbol (*) as a superscript in this text. For example, in lane 1 the plasmid DNA, separated by agarose gel electrophoresis and stained with ethidium bromide, isolated from A. tumefaciens strain A277 is shown as two horizontal migrating bands. The resulting autoradiogram in lane 1^* indicated that no hybridization with ^{32}P-labeled nif genes took place with the plasmids of A. tumefaciens strain A277. The lack of hybridization was expected as A. tumefaciens does not contain nitrogen fixation genes. The use of A. tumefaciens strain A277 is therefore a suitable negative control for the hybridization assays and also serves as a molecular weight standard for the plasmids of the Rhizobium strains.

An examination of the fast-growing Rhizobium in Table 1 shows

Table 1. Properties of large plasmids

Strain of origin	Plasmid(s)	Mwt. in Mdal[a]	Hybrid. to *nif*
R. japonicum (slow-growing type)			
61A76	pRja61A76	178 ±4	-
AA102	pRjaAA102	138 ±6	-
3I1b31	pRja3I1b31	160 ±11	-
3I1b71a	pRja3I1b71a	164 ±2	-
3I1b74	pRja3I1b74	195 ±6	-
3I1b94	pRja3I1b94a	58 ±9	-
	pRja3I1b94b	118 ±6	-
3I1b110		(none)	N. D.
3I1b143	pRja3I1b143	159 ±6	-
R. japonicum (fast-growing type)			
PRC191	pPRC191a	69	-
	pPRC191b	195	+
PRC193	pPRC193	186	+
PRC194	pPRC194a	76 ±13	-
	PPRC194b	145 ±5	-
PRC201	pPRC201a	117 ±4	-
	pPRC201b	192 ±25	+
PRC205	pPRC205a	57 ±15	-
	pPRC205b	112 ±3	+
	pPRC205c	192 ±25	-
PRC206	pPRC206a	54 ±12	-
	pPRC206b	60 ±8	-
	pPRC206c	197 ±27	+
R. meliloti			
102F28	pRme102F28a	73 ±4	-
	pRme102F28b	118 ±3	-
102F51	pRme102F51	93 ±3	+
R. phaseoli			
DB1	pRphDB1a	166	-
	pRphDB1b	248	+
A. tumefaciens			
A277	pTi	119 ±3	-
	Cryptic	300	-

[a]Molecular weights by Russell and Atherly (1981).

that structural nif genes are mostly located on one of the large plasmids in each strain. R. meliloti strain 102F28 (lane 2) contains two plasmids with molecular weights of 73 and 118 Mdal. The hybridization result (lane 2*) indicated that neither of these plasmids contain nif genes on the endogenous plasmids. However, R. meliloti strain 102F51 (lane 3) does contain nif genes on a 93 Mdal plasmid as can be seen in the autoradiogram in lane 3*. R. phaseoli strain DB1 harbors its nif genes on the 248 Mdal plasmid (lanes 4 and 4*). Of all the large plasmids examined in this study, pRphDB1b represents the largest plasmid which contains nif genes. Structural nif genes are apparently found on plasmids as large or larger in other R. phaseoli strains (D. Berryhill, Dept. of Microbiology, North Dakota State University).

The fast-growing R. japonicum contain nif genes on plasmids ranging in size from 112 to 197 Mdal and one strain harbors the nif genes on the chromosome or a megaplasmid. Strain PRC194 does not contain nif genes on either the 76 or 145 Mdal plasmid (lanes 5 and 5*). In this particular strain, the nif genes reside on a megaplasmid (not isolated by this procedure) or the chromosome. Nif genes are located on the large plasmid in strain PRC201 (lane 6 and 6*), strain PRC206 (lanes 8 and 8*), strain PRC193 (lanes 11 and 11*), and strain PRC191 (lanes 12 and 12*). The size of these large plasmids is fairly constant and ranges from 186 in strain PRC193 to 197 Mdal in strain PRC206. The location of nif genes on the 112 Mdal plasmid in strain

PRC205 is rather surprising (lanes 7 and 7*). This strain contains a 192 Mdal plasmid which is in the same size range as the large plasmids in the other fast-growing R. japonicum which did contain nif genes. Since this plasmid did not hybridize with the nif probe, it is apparent a plasmid of this size can reside in the fast-growing strains without structural nif genes.

The slow-growing R. japonicum strains do not contain nif genes on endogenous plasmids. Eight strains were examined for plasmids containing nif genes and negative results were obtained in each case. The results of nif hybridization to strains 61A76 and AA102 are shown in Figure 1. Neither the 178 Mdal plasmid pRja61A76 of strain 61A76 (lanes 9 and 9*) or the 138 Mdal plasmid pRjaAA102 of strain AA102 (lanes 10 and 10*) show any hybridization to structural nif genes. In order to confirm the validity of the Southern hybridization assays, colony hybridizations were performed with the total DNA of all the strains in Table 1. The total DNA is simply a lysate of the bacterial cells on the nitrocellulose filters used in the colony hybridization assays. It contains all plasmid, megaplasmid (if present), and chromosomal DNA. The slow-growing R. japonicum strains showed positive hybridization with the pRmR2 probe which indicated the expected relatedness with the nif genes of R. meliloti. An additional slow-growing R. japonicum strain, 3I1b110, which does not contain a readily isolatable plasmid, showed positive hybridization. A positive hybridization signal was also obtained with the fast-growing R.

japonicum strain PRC194 which contains two plasmids that did not hybridize by the Southern procedure. R. meliloti strain 102F28, which also did not hybridize with the nif probe to its two endogenous plasmids, showed a positive signal with the nif probe. The negative control in the colony hybridizations was the total DNA of A. tumefaciens; as expected, no hybridization took place. A possibility existed, however, that the positive hybridization was due to the vector of pRmR2. The vector is pACYC184 (Chang and Cohen, 1978) and is commonly used as a cloning vector due to its small size of 4.2 Kb and limited restriction sites. A control hybridization using pure pACYC184 was performed with an identical set of colony hybridization filters containing total DNA of all the strains listed in Table 1. Included in the hybridization as a positive control was pure pRmR2 on each nitrocellulose filter. The only positive hybridization occurred with pRmR2 which contained pACYC184 as a vector. This result confirmed the expected hybridization to the Rhizobium strains with the nif probe.

The nif hybridization results with the slow-growing R. japonicum agree with that reported by Haugland and Verma (1981) for two strains common to both studies. Strains 61A76 and 3I1b110 contained nif genes on a megaplasmid or the chromosome, and an endogenous plasmid up to 300 Mdal could not be found in strain 3I1b110, which is the same as that found in this study. Jouanin et al. (1981) did not find hybridization of pSA30, containing the structural nif genes from K. pneumoniae, with the 93 Kb plasmid from R. meliloti strain 102F51.

This result is in conflict with the data in this study concerning the hybridization of *nif* to the plasmid from *R. meliloti* 102F51 as shown in lanes 3 and 3* of Figure 1. The strength of the hybridization signal indicated a significant amount of homology exists. This is expected in that the *nif* probe originated from *R. meliloti* as described by Ruvkun and Ausubel (1980).

2. Plasmids in other *Rhizobia* species

An interesting phenomenon in both *Rhizobium* and *Agrobacterium* species is the predominance of large plasmids. Nuti et al. (1977) reported renaturation kinetic data of plasmids ranging from 70 to 400 Mdal in various slow- and fast-growing *R. japonicum* strains. Effective and ineffective strains were compared in *R. leguminosarum* and *R. trifolii* of which plasmid DNA was found in each type of strain. Although a direct correlation could not be made based on this initial study, Nuti et al. (1977) suggested the role of plasmid DNA in the symbiosis process. The large size of the plasmids in *Rhizobium* and the differences in cell wall composition significantly inhibited the isolation of the plasmids by methods developed for *E. coli* plasmids. Therefore, major modifications were introduced such that the large plasmids could be isolated from *Rhizobium* species. Plasmid isolation methods described by Currier and Nester (1976), Casse et al. (1979), and Hirsch et al. (1980) for use in *Agrobacterium* and *Rhizobium* greatly increased the yield of the large, fragile plasmids from these

species. However, much difficulty has been reported in the isolation of large plasmids from slow-growing R. japonicum relative to the fast-growing strains of Rhizobium (Gross et al., 1979; Hadley et al., 1983; Russell and Atherly, 1981). The plasmid isolation procedure described by Gross et al. (1979) could not be repeated by the authors (A. Vidaver, Dept. of Microbiology, Univ. of Nebraska, personal communication). Russell and Atherly (1981) were successful in the isolation of large plasmids from slow-growing R. japonicum using the method of Casse et al. (1979) and it was suggested a wash with 3% NaCl was responsible. As late as 1983 reports in the literature expressed difficulty in the isolation of plasmids from slow-growing strains (Hadley et al., 1983). The plasmid isolation methods used in this study with the fast- and slow-growing strains are described and discussed in detail in Section III.

Casse et al. (1979) demonstrated the presence of large plasmids in R. meliloti by using agarose gel electrophoresis (Meyers et al., 1976) in order to separate the large plasmids from chromosomal DNA. A significant advantage existed in this type of plasmid DNA determination in that Southern hybridizations could be performed with any DNA separated by agarose gel electrophoresis (Southern, 1975). The plasmid DNA of the Rhizobium could be probed for relatedness and the location of specific gene sequences. Therefore, the work of Nuti et al. (1977) was significant in determining the size of the large plasmids which could not be isolated by standard methods at that time.

But the agarose gel electrophoresis method of plasmid analysis by Casse et al. (1979) has had much more potential for genetic analysis and has been the method of choice in the study of plasmids from virtually all strains examined.

Molecular weight determinations of the large plasmids in R. meliloti were reported by Casse et al. (1979) and their size ranged from 90 to 200 Mdal. At least one large plasmid was present in 22 of 25 effective strains examined. Other Rhizobium species including R. leguminosarum and R. trifolii also contained plasmids which were isolated by gel electrophoresis. Contour length measurements made with electron microscope preparations of pure plasmid DNA from R. meliloti strain L5-30 estimated the single plasmid in this strain to be 91 ±2 Mdal. The gel electrophoresis results indicated a size of 89 ±3. The use of gel electrophoresis by Casse et al. (1979) instead of contour length measurements was therefore an accurate and easier plasmid molecular weight determinations. However, the molecular weights of plasmids greater than 140 Mdal were underestimated using gel electrophoresis when compared to contour length measurements. Casse et al. (1979) suggested the use of large plasmids with known molecular weights, determined by the contour length method, as molecular weight markers. The size of the plasmids isolated from Rhizobium and Agrobacterium in this study were compared to large plasmids from A. tumefaciens strain A277 as described by Russell and Atherly (1981). Courier and Nester (1976) determined the molecular weight of the Ti

plasmid to be 120 Mdal by contour length measurements. Although the 120 and 300 Mdal plasmids from the A. tumefaciens strain A277 were useful in determining molecular weights, the values shown in Table 1 are more likely an underestimation of the true molecular weights of the plasmids in R. japonicum. An example of the underestimation of the molecular weights comes from the addition of EcoRI fragments of the plasmid pPRC193 as described in Section III. The addition of all the fragments compared to bacteriophage lambda DNA standards was 214 Mdal. This is 28 Mdal larger than the gel electrophoresis determinations made by Russell and Atherly (1981).

Other studies have also determined that Rhizobium species contain large plasmids. The isolation and separation in agarose gels of large plasmids was first reported by Gross et al. (1979) and later by Russell and Atherly (1981) in both slow- and fast-growing strains of R. japonicum. A detailed explanation of the various plasmid isolation methods used may be found in Section III. Hirsch et al. (1980) described the isolation of large plasmids of R. leguminosarum by gel electrophoresis and found results similar to those of Casse et al. (1979) in that large plasmids are a common occurrence in R. leguminosarum. The loss of the largest plasmid in R. leguminosarum strain 6015, as shown by gel electrophoresis, resulted in the loss of nodulation ability. Therefore, two important conclusions may be drawn from the studies of plasmids in Rhizobium species. First, plasmids have been found in every species and in nearly every strain of

Rhizobium examined. Second, the size of the plasmids in the Rhizobium species is large: most plasmids are greater than 90 Mdal and megaplasmids (greater than 300 Mdal) have been examined.

The location of genes associated with symbiosis on large plasmids has been suspected since the original finding of the large plasmids in Rhizobium. Methods previously used to determine the location of nitrogen fixation and nodulation genes have included plasmid curing (Higashi, 1967; Zurkowski et al., 1973) and plasmid transfer (Brewin et al., 1980; Dunican and Tierney, 1974; Johnston et al., 1978). The success of these methods has been mixed and may readily be explained by the fact that genes involved in symbiosis may be located on the chromosome, and hence would not normally be transferred, and that known genetic markers on the plasmids are rare. One example of determining the plasmid location of nod genes was the transfer of a plasmid from R. phaseoli to a nodulation deficient R. leguminosarum strain. The R. leguminosarum strain containing the plasmid from R. phaseoli was able to nodulate peas, the host of the R. phaseoli (Lamb et al. 1982).

Several important studies convincingly determined the location of structural nif genes in various Rhizobium species by hybridization with a 32P-labeled nif probe. The nif probe was originally isolated from K. pneumoniae as previously described. Homology with nitrogen fixation genes in Rhizobium was expected based on the observation that protein subunits of the nitrogenase enzyme complex could be combined

from different sources and form an active protein complex and, also, that the amino acid content of nitrogenase proteins from a variety of sources were highly homologous (Nuti et al., 1979). Ruvkun and Ausubel (1980) clearly demonstrated the hybridization of the structural nif genes of K. pneumoniae with 19 of 19 nitrogen fixing organisms. Therefore, the use of nif genes from K. pneumoniae has provided an opportunity to examine the location of nif genes in the Rhizobia. The first study to determine the location of nif genes by hybridizations using separated DNA from Rhizobium was reported by Nuti et al. (1979). They demonstrated the hybridization of nif structural genes from K. pneumoniae and plasmid DNA from several R. leguminosarum strains and a strain of R. meliloti. Plasmid DNA was digested with a restriction enzyme and transferred to nitrocellulose by the Southern method. Second, the results of Hombrecher et al. (1981) showed hybridization with the K. pneumoniae nif probe and large plasmids in R. leguminosarum and R. phaseoli. Third, Prakash et al. (1981) also showed the presence of large plasmids in various Rhizobium strains and determined the location of structural nif genes on large plasmids in R. leguminosarum, R. trifolii, and R. phaseoli. In contrast, two R. meliloti strains examined by Prakash et al. (1981) contained plasmids which did not harbor nif genes. The nif genes in these strains were suspected of being located on a megaplasmid or the chromosome. Megaplasmids (larger than 300 Mdal) harboring nif genes have been reported in R. meliloti (Rosenberg et al., 1982) and have also been

detected in the fast-growing R. japonicum (M. J. Hagenson, Dept. of Genetics, Iowa State University, personal communication). In the slow-growing R. japonicum, however, megaplasmids have not been detected. (K. Engwall, Dept. of Genetics, Iowa State University, personal communication).

The results presented in this section are consistent with the results reported in the literature concerning the presence or absence of nif structural genes on large plasmids in Rhizobium. Because nif genes in slow-growing R. japonicum are located on the chromosome, it is important to design nif gene related experiments which account for this important factor. A distinct advantage exists in the knowledge of the location of nif genes in slow- and fast-growing R. japonicum. For instance, The large plasmids of the fast-growing strains which contain nif genes may be transferred to a slow-growing strain harboring nif genes on the chromosome or a megaplasmid. Such an experiment has been performed with the fast-growing strain PRC191 and slow-growing strain 3I1b110 (K. Engwall, Iowa State Univ., personal communication).

Significant differences exist between the slow- and fast-growing R. japonicum strains examined in this study. The distinct divergence in the location of structural nif genes is only one example. Biochemically, the slow-growing Rhizobium species are base producers in mannitol medium while the fast-growing Rhizobium species are acid producers in the same medium (Vincent, 1974). Agarwal and Keister (1983) have noted significant physiological differences within

slow-growing R. japonicum. Others have shown that fast-growing R. japonicum isolates from the People's Republic of China strongly resemble other Rhizobium fast-growing species both physiologically and biochemically (Keyser et al., 1982; Sadowsky et al., 1983). The fact remains, however, that both slow- and fast-growing R. japonicum strains are capable of nodulating the same soybean cultivar (Keyser et al., 1982; N. DuTeau, Dept. of Genetics, Iowa State University, personal communication).

Ruvkun and Ausubel (1980) have speculated that plasmid-borne, nitrogen-fixation related traits may be the result of recent radiation, perhaps by conjugative plasmids to other species. Therefore, it is possible that the fast-growing R. japonicum strains isolated from the People's Republic of China are relatively recent additions to the family of nitrogen fixing organisms. Alternatively, a fast-growing species of Rhizobium may have originally formed the symbiotic event. If fast-growing R. japonicum strain PRC194, with chromosomal-borne nif genes, is the original progenitor strain then the fast-growing type may be the ancestor. It may therefore prove to be relatively difficult in determining the original symbiont of the Rhizobium-soybean relationship.

III. CONSERVATION OF DNA SEQUENCES IN *RHIZOBIUM JAPONICUM*

A. Introduction

Rhizobium japonicum is the member of the family Rhizobiaceae that forms a symbiotic relationship with soybeans and fixes atmospheric nitrogen in root nodules. *R. japonicum* is of particular interest because of its symbiosis with the important agronomic crop, soybean, but also because of the recent discovery of fast-growing strains that appear to be unrelated to traditionally used U.S. strains (Keyser et al., 1982). The ability of both slow- and fast-growing *R. japonicum* strains to nodulate and fix nitrogen in a single soybean cultivar is unusual but not unique. Certain slow- and fast-growing *R. species*, which nodulate *Lotus pedunculatus* and the cowpea group, have also been identified (Brockwell, 1980). The physiological differences between slow- and fast-growing *R. japonicum* strains are significant (Keyser et al., 1982) and reflect a divergent genetic background. It has therefore been of interest to investigate the genetic content of *R. japonicum* in terms of sequence organization and conservation.

Large plasmids are a common feature in all *Rhizobium* species (Casse et al., 1979; Denarie et al., 1981; Gross et al., 1979; Nuti et al., 1977) and frequently contain genes of symbiotic importance (Masterson et al., 1982; Prakash et al., 1981; Rosenberg et al.,

1982). We have previously shown the presence of symbiotic genes on the plasmids of fast-growing R. japonicum, whereas slow-growing R. japonicum likely harbor these genes on the chromosome (Masterson et al., 1982).

In this study, we report the intra- and inter-relatedness between the plasmid and chromosomal DNA of both slow- and fast-growing R. japonicum strains. The structural organization of symbiotic genes appears highly conserved in the fast-growing R. japonicum strains isolated from diverse geographical and ecological backgrounds. However, in the slow-growing R. japonicum strains also of diverse backgrounds, such conservation is not as stringent. Nif structural gene sequences appear to be very highly conserved in all nitrogen-fixing organisms (Ruvkun and Ausubel, 1980), including the slow- and fast-growing R. japonicum strains examined here. In contrast, the slow-growing R. japonicum strains seemingly have very little homology with respect to nodulation gene sequences isolated from R. meliloti and also the fast-growing R. japonicum strains. Also, it is established that the nod and nif genes are present on the plasmids in fast-growing R. japonicum.

B. Materials and Methods

1. Bacterial strains and growth media

The properties of the R. japonicum strains used are summarized in

Table 1. For growth of cells for DNA isolation, late log-phase bacteria grown in TY-medium (Beringer, 1974) was used which contains per liter: 5g Bacto-Tryptone (Difco); 3g yeast extract; 0.93g $CaCl_2H_2O$. The bacteria were diluted 100-fold into PA-medium (Hirsch et al., 1980) which contains per liter: 4g Bacto-Peptone (Difco) and 0.24g anhydrous $MgSO_4$; and then grown to 2 X 10^8 cells/ml at 28°C with vigorous shaking. E. coli strains were grown in standard LB-medium containing per liter: 10 g Bacto-Tryptone (Sigma); 10 g NaCl; and 5 g yeast extract. E. coli were grown on a shaking platform at 37° C with appropriate antibiotic selection.

2. Isolation of total bacterial DNA

Late-log phase cells (5 ml) were centrifuged at 7,000 rpm in a Sorvall SS-34 rotor for 10 minutes at 5° C. The pellet was resuspended in 16 ml of 50 mM Tris-HCl, pH 8.0, 20mM EDTA, to which 10 mg/ml Sigma Co. Pronase E (final concentration 1 mg/ml) and 20% sodium dodecyl sulfate (final concentration 1%) were added. The mixture was incubated at 37° C for 2 h and the viscous solution was subsequently sheared twice through a 21 gauge needle. The solution was extracted with phenol and chloroform, and DNA precipitated by adding 3 M sodium acetate (final concentration 0.3 M) and 2.5 volumes of 95% ethanol. Total DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4° C, washed twice with 70% ethanol and resuspended in 0.5 ml 10 mM Tris, (pH 8.0) and 1.0 mM EDTA.

This relatively simple procedure works with great efficiency, is very rapid, and requires no bouyant density centrifugation. Samples resistant to restriction endonuclease digestion were re-extracted with phenol, chloroform, and ethanol as described above.

3. Isolation of plasmid DNA from Rhizobium japonicum

The relatively gentle plasmid isolation method of Hirsch et al. (1980) was used in the isolation of large plasmids from both slow- and fast-growing R. japonicum strains. Modifications were made in the procedure which resulted in increased yields, clean and completely digestible plasmid DNA, and ease of isolation. In particular, increased yields in plasmid DNA from slow-growing strains resulted from washing cells with cold 3% NaCl before lysis. This did not effect the isolation of plasmid DNA from fast-growing strains of R. japonicum. Thirty to fifty ug of high molecular weight plasmid DNA was usually isolated from one liter cultures of cells grown to a concentration of $2X10^8$ cells/ml.

Prior to cell lysis, one liter cultures of bacterial cells were washed with 50 mM Tris and 20 mM EDTA, pH 8.0 (referred to as high TE). The cells were resuspended in 160 ml of cold (4^oC) high TE and placed on ice in 500 ml plastic beakers. Ten ml each of predigested Pronase (Sigma) (10 mg/ml) and sodium dodecyl sulfate (10%w/v) were added to the resuspended cells and stirred with a plastic pipette to ensure complete mixing of all ingredients. Samples were placed in a

37° C waterbath such that the lysate levels in the beakers were equal to the water level in the waterbath. Over a period of 45 to 60 minutes the samples were occasionally stirred and the progress of cell lysis was monitored. Samples which were originally opaque and non-viscous usually turned clearer and very viscous within 60 minutes. Exactly 5.1 ml of fresh 3.0 M NaoH were added to each lysate sample and gently stirred with a plastic pipette over a 30 minute period in order to denature the DNA. The pH of the lysate was monitored with pH paper in order to determine the extent of denaturation. Fifteen ml of cold 2.0 M Tris, pH 7.0, were added and gently mixed with each sample. The chromosomal DNA, which is not supercoiled, mostly remained denatured while the supercoiled plasmid DNA was able to rapidly renature. This allowed the precipitation of chromosomal DNA (as well as SDS-protein complexes) in the next step of the procedure.

The addition of 55 ml of cold 5 M NaCl to each lysate initiated the precipitation process. Complete precipitation occurred overnight. Unless otherwise noted, all samples were kept at 4° C throughout the rest of the isolation procedure. Each sample, containing approximately 280 ml of lysate solution, was distributed into clean 150 ml Corex centrifuge tubes and centrifuged at 8,000 rpm for 20 minutes at 4° C. The supernatant, which contained mostly plasmid DNA with a limited amount of chromosomal DNA contamination, was carefully poured into clean, 500 ml plastic beakers. To the supernatant exactly 68 ml of 8,000 Mwt polyethylene glycol (50% w/v) were added to each sample and

gently stirred until complete mixing had occurred. This mixture was kept at 4^{o} C overnight and centrifuged using a GSA (Sorvall) rotor, with 150 ml Corex centrifuge tubes, at 7,000 rpm for 10 minutes at 4^{o} C. The supernatant mixture was carefully poured off and the Corex tubes were inverted on paper towels and allowed to dry over a period of approximately one hour. At this time, the tubes were placed on ice and 7.5 ml of sterile high TE (pH 8.0) were distributed among 3 Corex tubes which corresponded to a single lysate sample.

All plasmid DNA was purified in CsCl-ethidium bromide gradients to eliminate chromosomal contamination in subsequent hybridization assays. Exactly 7.80 g of CsCl (Sigma) was gently added and mixed with each sample over a 10 minute period at 4^{o} C. 0.5 ml of ethidium bromide (5 mg/ml) was then added to each sample and gently mixed. The refractive index of each sample was determined and any sample which significantly deviated from a value of 1.390 was corrected by adding either CsCl or sterile high TE (pH 8.0).

Each sample was carefully added to 12 ml capacity polypropylene ultracentrifuge tubes (Beckman) and sealed with a Beckman ultracentrifuge heat sealing device. The tubes were placed in a Ti50 ultracentrifuge rotor (Beckman) and centrifuged in a Beckman L-2 ultracentrifuge at 36,000 rpm for 48 hours at 15^{o} C. After this time, the timer on the ultracentrifuge was turned off and the rotor was allowed to come to a complete stop. The braking device on the ultracentrifuge was not used so that the gradients were not

significantly disturbed in the deceleration process. The tubes were carefully removed from the ultracentrifuge and wrapped in aluminum foil to protect each sample from light. Using an ultraviolet light source (Fotodyne), the tops of each ultracentrifuge tube were removed and the band containing chromosomal DNA, as well as open circular and linear plasmid DNA (top band), was removed using a sterile, narrow pipette and was then discarded. Another sterile, narrow pipette was used to remove the covalently closed circular plasmid DNA (lower band). The plasmid DNA solution was placed in a sterile 10 ml tube (Falcon) to which an equal volume of isopropanol, saturated with 5 M NaCl, was added and gently mixed with each sample and allowed to form two phases. The top phase contained extracted ethidium bromide in the saturated isopropynol solution. This step was repeated 4-5 times and then checked with the ultraviolet light source to ensure the removal of ethidium bromide. If ethidium bromide was still present, several more extractions were performed in order to remove it. Each sample, with a volume between 0.5 and 1.0 ml, was extensively dialized versus 10 mM Tris and 1 mM EDTA, pH8.0. Samples were routinely concentrated by dialysis versus 10 mM Tris (pH 8.0) and 50% polyethylene glycol at 4^{o} C. Finally, the concentrated samples were stored at 4^{o} C.

There are three main factors in the isolation procedure outlined above which greatly assist in the isolation of the large plasmids. First, all materials which could be sterilized by using an autoclave or by rinses with 95% ethanol were done prior to an isolation in order

to reduce nuclease contaminations. Second, the plasmid DNA only came into contact with plastic and not with glass, which may bind DNA, throughout the entire isolation process. Third, the plasmid DNA was removed from the CsCl-ethidium bromide gradients using plastic pipettes. The bore of the pipette is approximately 1 mm and this size is sufficient in order to reduce shear forces. While glass may be siliconized, this treatment has the adverse effect of not allowing DNA to adhere in precipitation reaction steps.

Furthermore, in the initial trials of this isolation method, it was valuable to take small aliquots at each stage in order to determine the presence or absense of plasmid DNA. The aliquots were loaded onto agarose gels, subjected to electrophoresis, and stained with ethidium bromide (described in part 5 of this section) so that the maximum amount of plasmid DNA could be isolated from each individual step. For instance, the addition of exactly 5.1 ml of 3 M NaOH in the denaturation step yielded the highest amount of plasmid DNA.

4. Isolation of plasmid DNA from E. coli

The small-scale isolation procedure of Birnboim and Doly (1979) was used in the extraction of small and medium sized plasmids. This procedure is rapid, repeatable, and up to 40 samples of plasmid DNA copuld be isolated and digested with restriction enzymes in a single day. However, this method of plasmid isolation is limited to E. coli

and was not effective in the isolation of plasmids from R. japonicum.

Five ml of L-media, which contains per liter: 10 g Bacto-Tryptone (Sigma), 5 g yeast extract, and 10 g NaCl, were used with the appropriate antibiotics for plasmid selection. A single colony of E. coli was inoculated into the L-media and allowed to grow overnight with vigorous shaking at 37° C. 1.3 ml of this culture were centrifuged for one minute in a 1.5 ml Eppendorf centrifuge tube using an Eppendorf centrifuge (Eppendorf). The supernatant was discarded and the bacterial pellet was resuspended in 100 ul of cold lysis solution (50 mM glucose, 10 mM EDTA, 4 mg/ml lysosyme, and 25 mM Tris (pH 8.0). The lysate mixture was allowed to stand at room temperature for 5 minutes after which 200 ul of cold denaturation solution (0.2 M NaOH, 1% sodium dodecyl sulfate) were added. The tube was inverted gently and placed on ice for 5 minutes. At this time, 150 ul of renaturation solution (3 M acetic acid) were added, gently but thoroughly mixed, and placed on ice for another 5 minutes. The lysate sample was then centrifuged in an Eppendorf centrifuge (Eppendorf) for 5 minutes and the supernatant was placed into a new 1.5 ml Eppendorf centrifuge tube. One volume of phenol, previously equilibrated with 1 M Tris (pH 8.0), was added to the supernatant and gently mixed. The sample was then centrifuged for 2 minutes and the top layer was carefully removed. An equal volume of chloroform was added, gently mixed, and centrifuged for 2 minutes. The top layer was removed and placed in a new 1.5 ml Eppendorf centrifuge tube to which 1/2 volume of 7.5 M

ammonium acetate and 2 volumes of -20° C ethanol were added and gently mixed together. The mixture was placed at -60° C for 10 minutes, allowed to reach 4° C, and centrifuged for 10 minutes at 4° C. The supernatant was discarded and 200 ul of 80% ethanol in water were used to rinse the DNA pellet. The sample was centrifuged for one minute, the supernatant was removed, and the DNA pellet was allowed to dry. Fifty ul of low TE (0.01 M Tris (pH 8.0), and 0.001 M EDTA) were added and gently but thoroughly mixed. DNA samples prepared in this manner could be stored at -20° C until needed.

Small aliqouts of 5 ul were applied to mini-gels (described in the preceding section) so that the concentration and size of the extracted plasmid DNA could be analyzed. Usually, the plasmid DNA prepared in this manner could be readily digested with any given enzyme. If a sample of plasmid DNA was resistant to digestion, the isolation procedure was repeated from the phenol step. This usually solved problems associated with digestion by restriction enzymes.

In order to obtain 20-30 ug of plasmid DNA from E. coli, the isolation procedure of Birnboim and Doly (1979) was scaled up 20 fold. CsCl bouyant density centrifugation, as described in part 3, was carried out in exactly the same manner. It was necessary to purify all plasmid DNA samples prior to nick-translation reactions such that chromosomal DNA was not incorporated in the labeling process. Furthermore, nick-translation labeling reactions were optimum only with purified DNA.

5. Restriction enzyme analysis and electrophoresis conditions

R. japonicum plasmids and total DNA were routinely digested with 10 units of EcoRi restriction enzyme (New England Biolabs) per ug of DNA for 2 h at 37° C. High salt restriction enzyme buffer (100 mM NaCl, 50 mM Tris (pH 8.0), 10 mM MgCl, and 1 mM dithiothreitol) was used for digestions with EcoRI and was prepared as a 10X stock, filter sterilized, and kept at -20° C. After digestion, the samples were placed at 65° C for 10 minutes and then on ice until needed.

Agarose electrophoresis was a technique routinely used for two purposes. First, different sized fragments, ranging from less than 1 kb to over 35 kb, could be efficiently separated and thus characterized by unique digestion patterns. Second, the separated DNA could be transferred to nitrocellulose paper such that DNA:DNA hybridizations could be performed in order to determine relatedness at the DNA level. The concentration of agarose for most gels, in which fragments of DNA were separated, was optimum at 0.7%. Several different types of electrophoresis buffers were tried of which TAE buffer (0.04 M Tris-acetate (pH 8.0), 0.002 M EDTA) was superior. Other buffers which contained boric acid sometimes gave the phenomena of lane constriction in which the migrating tract of DNA would become narrower as electrophoresis progressed.

Samples were routinely loaded onto a 300 ml 0.7% agarose gel (Seakem), made in TAE buffer. The powdered agarose was added to TAE

buffer and brought to a boil on a hot plate (or a microwave) and allowed to cool to 60° C before being poured into an electrophoresis agarose holding tray with a comb (Bethesda Research Labs) providing 1 mm thick wells which could hold approximately 20 ul of DNA solution. Electrophoresis was performed at 3 V/cm for 18 h at room temperature with a horizontal apparatus (Bethesda Research labs) which contained TAE buffer. After electrophoresis was complete, the gel was submerged in distilled water containing 1 mg/ml of ethidium bromide (5 mg/ml stock solution). The agarose gel was then rinsed with distilled water and exposed to ultraviolet light using a 300 nm ultraviolet light source (Fotodyne). Photographs were taken of the gel using a Polaroid MP4 apparatus and Polaroid 665 film. A gelatin filter (Kodak) under a Kodak Wratten 23 filter beneath the lens of the camera provided adequate filtration for proper exposure of the film. Lambda bacteriophage DNA (Bethesda Research Labs) digested with EcoRI or HindIII (New England Biolabs) was included in all experiments as molecular weight determinants and for negative controls in subsequent Southern hybridizations. Several digestions were done with R. japonicum DNA and band patterns compared to determine that complete digestion occurred.

The mini-gel apparatus described by Maniatis et al. (1982) was used to determine the concentration and extent of restriction endonuclease digestion. This allowed one-tenth the amount of DNA required for a full sized gel to be analyzed in approximately 30

minutes when subjected to the same electrophoresis conditions described above. These gels were also made of 0.7% agarose (Seakem) in TAE buffer (0.04 M Tris-acetate (pH 8.0), 0.002 M EDTA). The agarose in the TAE buffer were melted on a hot plate (or microwave) to boiling and allowed to cool to 50-55^{o} C before casting mini-gels. Two by three inch glass microscope slides were used as a platform to which approximately 10 ml of molten agarose were added. The use of mini-gel's afforded the convenience of determining whether or not the DNA had completely digested. If the DNA was not satisfactorily digested, the procedure could be repeated again and rechecked on a mini-gel without significant loss of DNA. Also, the relative intensities of stained DNA in the mini-gels allowed an estimation of the amount of DNA present. This information was used to determine the amount of digested DNA that should be added to a full sized gel so that a uniform amount was applied to each lane. This factor was significant when comparing hybridization patterns between the DNA isolated from various strains.

6. Hybridization conditions

The Southern (1975) procedure, as modified by Thomashow et al., (1980) was used in the preparation and blotting of suitable gels. Gene Screen (New England Nuclear) was used as a transfer membrane such that repeated hybridizations could be performed with a single blot. Hybridizations were performed in plastic pouch type bags at 42^{o} C for

48 h in a shaking water bath in formamide hybridization solution (50% formamide, 0.2% polyvinyl-pyrrolidine, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris (pH 7.5), 0.1% sodium pyrophosphate, 1.0% sodium dodecyl sulfate, and 100 ug/ml of denatured salmon sperm DNA). After 12-15 hours of hybridization, ^{32}P-labeled probe (final concentration approximately 10 ng/ml) were added in an additional 2 ml of the formamide hybridization solution and the hybridization reaction was allowed to proceed for 36 hours. When the hybridization period was complete, the hybridization fluid was removed from the plastic bag and three washes were performed. First, 100 ml of wash solution number one (0.3 M NaCl, 0.06 M Tris (pH 8.0), 0.002 M EDTA) were added to the hybridization bag and was placed in a shaking waterbath at room temperature for 5 minutes after which the wash solution was discarded; this step was repeated once. Next, 100 ml of wash solution number two (0.3 M NaCl, 0.06 M Tris (pH 8.0), 0.002 M EDTA and 1% sodium dodecyl sulfate) were added and the bag was placed at 60^{o} C in a shaking waterbath for 30 minutes; this step was repeated once. Third, 100 ml of wash solution three (0.006 M Tris (ph 8.0), 0.0002 M EDTA) were added and placed in a shaking waterbath at room temperature for 30 minutes; this step was also repeated once. At this time, the hybridization filter was examined with a hand-held Geiger counter (Lionel) in order to determine the amount of background radioactivity. If significant levels remained, then 1-2 washes with wash solution number 3 were repeated.

Both positive and negative controls reacted as expected under the

stringency of the hybridization conditions and are described in the results and discussion section of this chapter. ^{32}P-labeled dCTP and nick-translation chemicals were obtained from New England Nuclear and 0.5 to 1.0 ug of DNA probe was labeled to a specific activity between $5x10^6$ to $1x10^7$ cpm. Hybridized filters were exposed to Kodak X-Omat R film for 1-5 days with or without a Dupont Cronex Lightning-Plus intensifying screen (Dupont). To reuse DNA blotted hybridization filters, each filter was first washed with 60% formamide, 2X SSC (1X SSC: 0.15 M NaCl, 0.015 M NaCitrate), at 70^o C for two hours and then washed twice with 2X SSC at room temperature. Filters were exposed to film overnight with an intensifying screen to confirm the removal of ^{32}P-probe. Reduced stringency conditions were performed at 37^o C for 48 h in 10X Denhardts solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin), 3X SSC, 0.5% SDS, and 35% deionized formamide. Hybridization washes were done with 3X SSC and 3X SSC plus 0.5% SDS. Exposure to X-ray film is exactly as described above.

C. Results and Discussion

1. Isolation of *R. japonicum* plasmid DNA

The isolation of plasmid DNA from slow-growing *R. japonicum* is relatively difficult when compared to the isolation of plasmids from

other Rhizobium species and other gram negative bacteria in general. There are two significant reasons why the plasmids are resistant to standard isolation techniques. First, the plasmids are very large in size: most plasmids in slow- and fast-growing R. japonicum are over 100 Mdal (1.6 K bp) and most strains contain plasmids with an average molecular weight of approximately 175 Mdal, as described in the previous section). Most isolation procedures involve a cleared lysate process and are applicable to plasmids no larger than approximately 30 Mdal. Second, the slow- growing R. japonicum are very resistant to cell lysis. Russell and Atherly (1981) modified an isolation procedure used on fast-growing Rhizobia developed by Casse et al., (1979) which included washing the cells with 3 % NaCl prior to cell lysis. It was suggested the salt wash aided in the removal of substances on the cell wall (possibly polysaccharides) which inhibited cell lysis. A report by Schwinghamer (1980) stated that the use of sarkosyl detergent (0.1%) as a wash prior to cell lysis of gram negative bacteria was useful in increasing yields of plasmid DNA. In this study, washes with sarkosyl (0.1%) or sodium dodecyl sulfate prior to cell lysis of slow-growing R. japonicum did not appear to improve the quality or quantity of plasmid DNA.

Two general plasmid isolation procedures work well in the isolation of large plasmids from R. japonicum. Both procedures use alkaline denaturation and CsCl gradients. The first procedure, developed by Currier and Nester (1976), uses SDS and pronase to lyse

the bacteria, after which the lysate is sonicated and denatured. The lysate solutions are then neutralized, extracted with phenol, and finally ethanol precipitated. The isolation procedure of Casse et al., (1979) is very similar and slight modifications have been made so that the optimum amount of plasmid DNA could be isolated from fast-growing Rhizobium. The plasmid isolation of Adachi and Iyer (1980) is also very similar to the Currier and Nester (1976) procedure and was modified for use with plasmids of R. meliloti. The second general isolation technique for large plasmids was developed by Hanson and Olsen (1978) for the isolation of large plasmids (100-200 Mdal) from various Psuedomonas strains. This procedure is significantly different from that of Currier and Nester (1976) in three ways: 1) alkaline denaturation occurred after cell lysis (not during lysis); 2) phenol and chloroform extractions were omitted; and, 3) precipitation of plasmid DNA at the end of the procedure was done with polyethylene glycol (Mwt 6000) instead of ethanol. The use of polyethylene glycol in the isolation of plasmid DNA was originally reported by Humphreys et al. (1975). Plasmids ranging from 6-123 Mdal were isolated by a simple cleared lysate procedure followed by precipitation with various concentrations and molecular weights of polyethylene glycol. It was determined that a minimum final concentration of 10% and molecular weight of 6000 for polyethylene glycol was optimum in the isolation of large plasmids. Furthermore, it was reported that higher yields were possible and that polyethylene glycol was a better reagent for the

precipitation of plasmid DNA than ethanol.

While the large plasmids of slow-growing R. japonicum have been separated on large gels (Gross et al. 1979; Masterson et al. 1982; Russell and Atherly, 1981) the amount of plasmid and quality of the DNA isolated by the procedures described by the authors varied according to the procedure used. The procedure described by Gross et al. (1979) for the isolation of plasmid DNA from slow-growing strains was unsuccessful in this particular study. The isolation technique described by Casse et al. (1979) and used in essentially the same form by others (Berry and Atherly, 1984; Haugland and Verma, 1981; Masterson et al. 1982; Russell and Atherly, 1981) with the plasmids of slow-growing R. japonicum has been useful in isolating sufficient amounts of plasmids which could be analyzed with restriction endonucleases.

In this study, the plasmid isolation procedure developed by Hirsch et al. (1980) for large plasmids in R. leguminosarum was used in the isolation of plasmids from slow- and fast-growing R. japonicum strains. This procedure uses the cell lysis step of the Currier and Nester (1976) method, denaturation after cell lysis and use of polyethylene glycol for plasmid precipitation according to the Hanson and Olsen (1978) method. Phenol and chloroform extractions have also been omitted. Comparatively, this procedure yields the highest concentration of plasmid DNA of any method used in this study. One liter of R. japonicum cells usually produced 30-40 ug of plasmid DNA

by this method (described in part 3 of Materials and Methods section). The plasmid DNA was of a very pure form without chromosomal DNA or protein contamination as may be seen in Figures 1 and 2. The importance of obtaining pure DNA was not only for digestion with restriction endonucleases and subsequent gel electrophoresis, but also for Southern hybridizations. Quality hybridizations with plasmid DNA were only obtained with pure DNA transferred to nitrocellulose filters and with pure DNA used in ^{32}-labeling reactions.

2. Intra-relatedness of plasmids in slow-growing strains

Table 1 lists the properties, including the source and serogroup of each strain, of the slow- and fast-growing R. japonicum strains used in this study. The serogroups were reported by Keyser and Weber (1979) for the slow-growing strains of R. japonicum but, however, no information regarding the serogroups of the recently introduced fast-growing strains of R. japonicum from the People's Republic of China is available. The molecular weights of the plasmid(s) in each strain are located in Section II.

The homology between plasmids of eight different slow-growing strains was estimated by restriction digestion patterns and hybridization analysis (Figure 1A and 1B). A significant amount of sequence conservation exists between the plasmids of four different isolates as can be seen by comparing the digest pattern of the plasmids in lanes 1-4. These plasmids were isolated from strains

Table 1. Properties of R. Japonicum strains

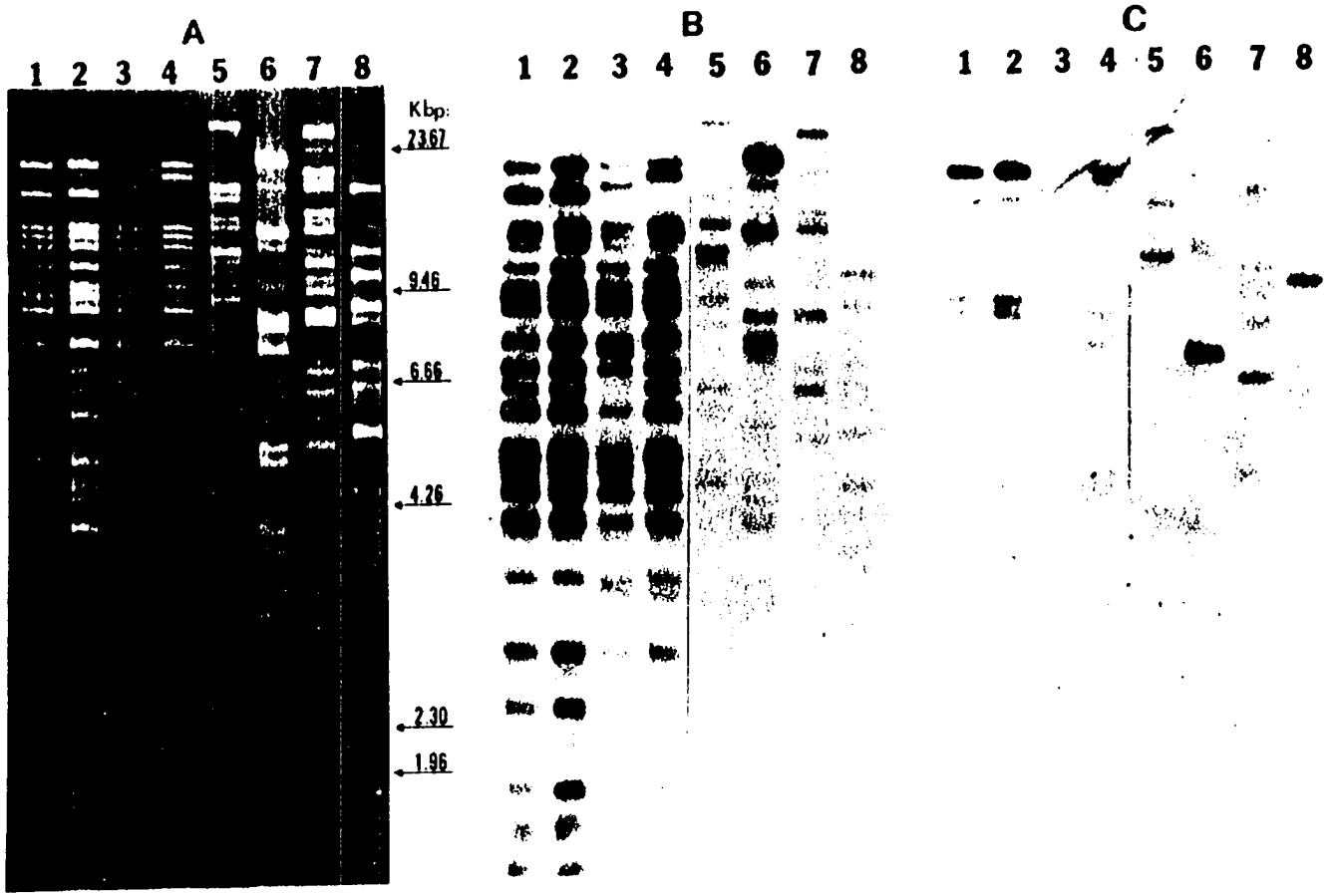
Strain	Source	Serogroup
R. japonicum (Slow-growing type)		
61A76	Mississippi, 1943	c3
Aa102	Iowa,1979	N.D.
3I1b31	Wisconsin, 1941	c3
3I1b71a	Arizona, 1948	c2
3I1b74	California, 1956	76
3I1b94	North Carolina, 1956	94
3I1b110	Florida, 1959	110
3I1b123	Iowa, 1960	123
3I1b143	India, 1973	110-143
(Fast-growing type)		
PRC191	People's Republic of	N.D.
PRC193	China: East Central	N.D.
PRC194	Provinces of Shansi,	N.D.
PRC201	Honan, Shandong, and	N.D.
PRC205	Shanghai.	N.D.
PRC206		N.D.

[a]From Keyser and Weber, 1979.

3I1b31, 61A76, 3I1b71a, and 3I1b143, which were originally isolated from diverse geographical and ecological backgrounds (Wisconsin, Mississippi, Arizona, and India, respectively). The plasmids in strains 3I1b31 and 61A76 (lanes 1-2) are identical with respect to EcoRi digestion patterns and are in agreement with the data of Berry and Atherly (A. G. Atherly, Iowa State University, personal communication); the plasmids from strains 3I1b71a and 3I1b143 show only minor differences. The EcoRI restriction digest patterns of plasmid DNA from the four other strains examined (Figure 1, lanes 5-8) are distinctly different from each other as well as from the digests of plasmids in lanes 1-4.

Since plasmid pRja61A76 is highly conserved in the four strains examined, it was chosen as a representative for hybridization assays between plasmids of slow-growing strains. A Southern blot of the DNA fragments shown in Figure 1A was hybridized with ^{32}P-labeled pRja61A76. The resulting autoradiogram is shown in Figure 1B. As expected, complete homology occurred among pRja61A76 and the three plasmids that showed nearly identical restriction patterns (Figure 1, lanes 1-4). In contrast, a limited amount of homology is seen between pRja61A76 and the plasmids in Figure 1, lanes 5-8. The bands which do show homology in these lanes do not correlate with similar-sized bands, with each other, or with the probe pRja61A76. Consequently, it seems that the plasmids in lanes 5-8 are not closely related to pRja61A76 (or to plasmids identical to pRja61A76) and thus contain

Figure 1. Gel electrophoresis of 1 ug of EcoRI digested plasmids from slow-growing strains A: Lane 1, pRjapI1b31; lane 2, pRja61A76; lane 3, pRja3I1b71a; lane 4, PRja3I1b143; lane 5, pRja3I1b74; lane 6, pRja3I1b94a,b; lane 7, pRja3I1b123; lane 8, pRjaAA102. Molecular weight markers are bacteriophage lambda digested with Hind III. B: Hybridization of ^{32}P-pRja61A76 to a Southern blot of the gel in Figure 1A; lane contents are the same as Figure 1A. C: Hybridization of ^{32}P-pRjaPRC193 to the same Southern blot in B after the probe in B was removed (as described in the Materials and Methods)


A
1 2 3 4 5 6 7 8
Kbp:
23.67
9.46
6.66
4.26
2.30
1.96
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8

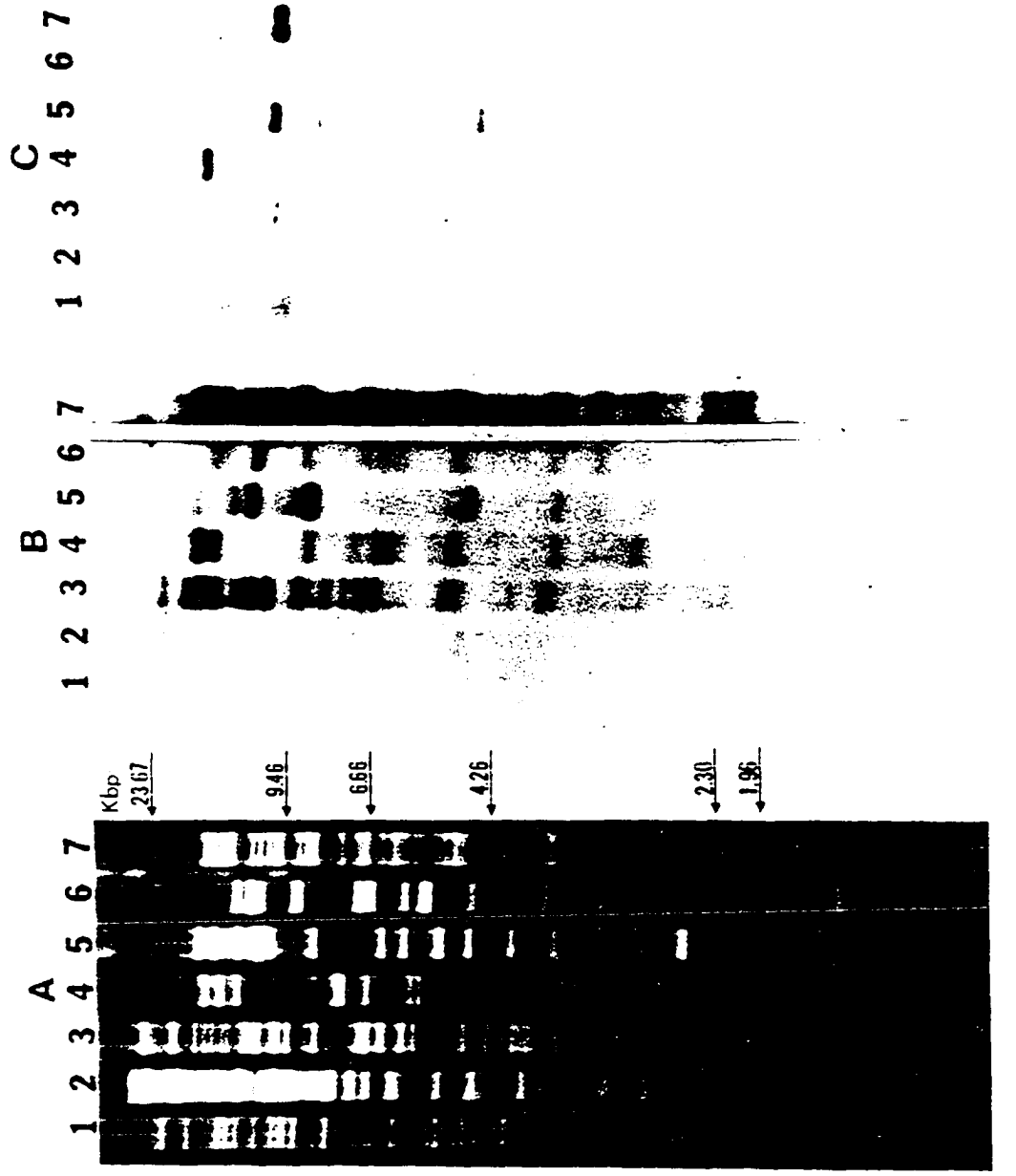
significantly different DNA sequences.

3. Intra-relatedness of plasmids in fast-growing strains

Figure 2A shows the gel electrophoresis pattern of EcoRI-digested plasmids isolated from six different fast-growing strains obtained from different locations in the People's Republic of China. Each strain contains 1-3 large plasmids (Masterson et al., 1982) and each strain has a comparatively unique restriction digest pattern. Variable amounts of the largest plasmid obtained from each strain exists because of plasmid fragility and loss in CsCl gradients. EcoRI-digested plasmid pRja61A76 DNA (from slow-growing strain 61A76) was placed in lane 1 to compare band patterns and for subsequent hybridization assays. A visual comparison of the digest pattern of pRja61A76 DNA and the plasmids from the fast-growing strains indicates no distinct conservation of restriction sites.

The only fast-growing R. japonicum strain in this study that contains a single plasmid (other than a very large megaplasmid, not separated by the isolation method used) is PRC193. Therefore, the 214 Mdal plasmid pRjaPRC193 was chosen as a hybridization probe. Figure 2B is a Southern blot of the DNA fragments hybridized to ^{32}P-labeled pRjaPRC193. Even though the restriction pattern between the plasmids of fast-growing strains appears variable, conservation of many similar sized fragments exist. This relatedness is particularly evident between the labeled plasmid (pRjaPRC193) and the plasmid(s) in strains

Figure 2. Gel electrophoresis of 1 ug of EcoRI digested plasmids from a slow-growing strain, pRja61A76 (lane 1) and from fast-growing strains (lanes 2-7) A: Lane 1, pRja61A76; lane 2, pRjaPRC194a,b; lane 3, PRjaPRC201a,b; lane 4, pRjaPRC205a,b,c; lane 5, pRjaPRC206a,b,c; lane 6, pRjaPRC191a,b; lane 7, pRjaPRC193. Molecular weight markers are bacteriophage lambda DNA digested with HindIII. B: Hybridization of ^{32}P-pRjaPRC193 to a Southern blot of the gel in A; lane content is the same as A. C: Hybridization of ^{32}P-pRja61A76 to the same Southern blot in B after the probe in B was removed (as described in the Materials and Methods section). The contents of lanes 1 and 2 were deliberately underexposed in order to identify individual bands of pRja61A76 for molecular weight determinations (see text for explanation).


C
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
A
1 2 3 4 5 6 7
Kbp
23.67
9.46
6.66
4.26
2.30
1.96

PRC201, PRC205, PRC206, and PRC191, (lanes 3-6 of Figure 2B). The digest of the two large plasmids from PRC194, which do not harbor nif structural genes (Masterson et al., 1982), showed very limited homology to the labeled plasmid (lane 2), as does the plasmid pRja61A76 (lane 1) from the slow-growing R. japonicum strain 61A76.

4. Inter-relatedness in slow- and fast-growing strains

Cross-hybridization between plasmids isolated from both slow- and fast-growing strains was done in order to determine how closely related the plasmids were to each other. The results of these hybridizations are shown in Figures 1C and 2C. ^{32}P-labeled pRjaPRC193 was hybridized to the Southern blot of the DNA digest seen in Figure 1A (which was stripped of previous ^{32}P-probe) and resulted in the autoradiogram shown in Figure 1C. Identical bands common to the labeled probe exist in the four similar lasmids in Figure 1C, lanes 1-4. Hybridization also occurred with the plasmids in lanes 5-8, although no similar-sized band was common between these four different plasmids.

Homology of ^{32}P-labeled pRja61A76 to the plasmids of fast-growing strains is shown in the autoradiogram in Figure 2C. The labeled plasmid from the slow-growing strain (61A76) hybridized to a limited number of bands in lanes 3-6, whereas the plasmids in strain PRC194 showed no significant hybridization. Note that the EcoRI digest of plasmid pRja61A76 in Figure 2C, lane 1, was deliberately underexposed

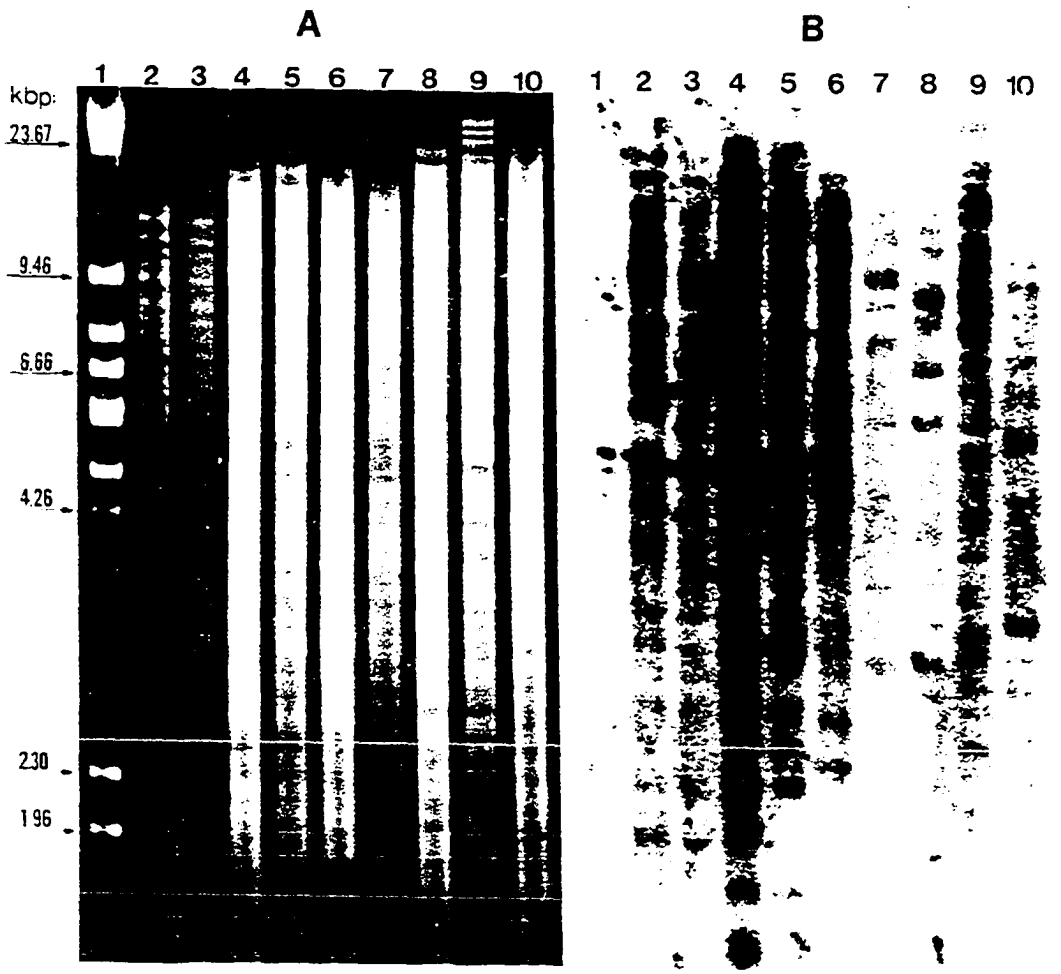
to identify individual bands for molecular weight determinations. Although the plasmid digests of strain PRC194 in Figure 2C, lane 2, are also underexposed, no significant increase in hybridization signals occurs with longer exposures.

The significance of the homologous DNA is not known at this time. It is possible that similar plasmid DNA sequences, such as an origin of replication, are common between the plasmids of fast- and slow-growing R. japonicu strains. However, the fact that such a limited amount of plasmid DNA between the two types of R. japonicu are homologous suggests that few genes are common.

5. Conservation of pRja61A76 in slow-growing strains

To ascertain if plasmid DNA sequences occurred in total DNA of slow-growing strains, ^{32}P-labeled pRja61A76 DNA was hybridized to a Southern blot of EcoRI-digested total DNA isolated from nine different slow-growing Rhizobium japonicum strains. The resulting autoradiogram is shown in Figure 3. The hybridization of ^{32}P-pRja61A76 to total DNA of all slow-growing strains examined is the same as when the probe is hybridized to the digestion pattern of the plasmid isolated from each strain. Strain 3I1b123 has additional bands, which hybridize in common with sequences on pRja61A76 (lane 9 of Figure 3B). Consequently, it would seem that the DNA present in the plasmid of strain 61A76 is highly conserved but not always present in plasmid form in other strains.

Figure 3. Gel electrophoresis of 1-2 ug of EcoRI digested total DNA from slow-growing strains A: Lane 1 contains bacteriophage lambda DNA digested with HindIII (molecular weights shown in kb) and EcoRI (molecular weights not shown). Lane 2, 3I1b31; lane 3, 61A76; lane 4, 3I1b71a; lane 5, 3I1b143; lane 6, 3I1b74; lane 7, 3I1b94; lane 8, 3I1b110; lane 9, 3I1b123; lane 10, AA102. B: Hybridization of ^{32}P-pRja61A76 to a Southern blot of the gel in A; lane order is the same.

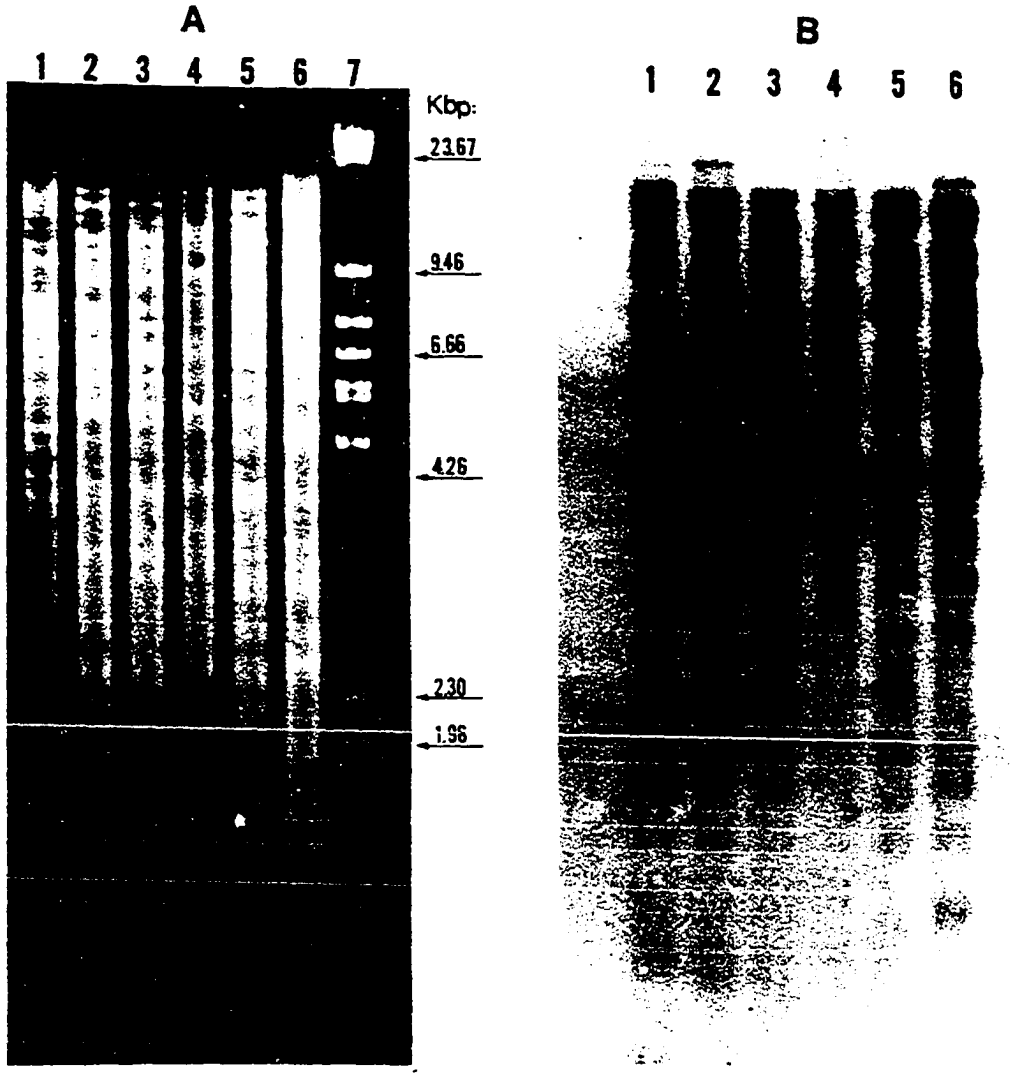

A
B
1 2 3 4 5 6 7 8 9 10
kbp:
23.67
9.46
6.66
4.26
2.30
1.96
1 2 3 4 5 6 7 8 9 10

Our examination of the slow-growing strain 3I1b110 has shown that this strain does not harbor a plasmid (other than a possible megaplasmid not isolated using the present procedure). However, the labeled plasmid from the slow-growing strain 61A76 hybridized with a number of bands of total DNA from 3I1b110 (lane 8 of Figure 3B). Furthermore, several bands that hybridize are approximately the same size as the corresponding fragments of pRja61A76. This suggests a conservation of plasmid sequences and are in agreement with the study of Haugland and Verma (1981).

6. Conservation of pRjaPRC193 in strain PRC194

Because little sequence homology exists between the plasmids in fast-growing strain PRC194 and PRC193 (Figure 2B, lane 2), it was of interest to determine if any plasmid sequences were conserved in the chromosome or a megaplasmid in this particular strain. Figure 4 shows the autoradiogram obtained after EcoR1-digested total DNA from fast-growing strains was hybridized to ^{32}P-labeled pRjaPRC193 plasmid DNA. Rather surprisingly, pRjaPRC193 plasmid DNA sequences are present in strain PRC194 in either a megaplasmid or in the chromosome (Figure 4, lane 6). Other fast-growing strains examined in this study also have a very high degree of sequence conservation with the labeled probe (Figure 4, lanes 1-4). A comparison of the hybridization of ^{32}P-labeled pRjaPRC193 with total DNA in Figure 4 and with plasmid DNA in Figure 2 shows a significant increase in hybridization to total DNA

Figure 4. Gel electrophoresis of 1-2 ug of EcoRI-digested total DNA from fast-growing strains A: Molecular weight markers are bacteriophage lambda (lane 7) as described in Figure 3. Lane 1, PRC201; lane 2, PRC205; lane 3, PRC206; lane 4, PRC191; lane 5, PRC193; lane 6, PRC194. B: Hybridization of ^{32}P-pRjaPRC193 to a Southern blot of the gel in A; lane content is the same.


A
1 2 3 4 5 6 7
Kbp:
23.67
9.46
6.66
4.26
2.30
1.96
B
1 2 3 4 5 6

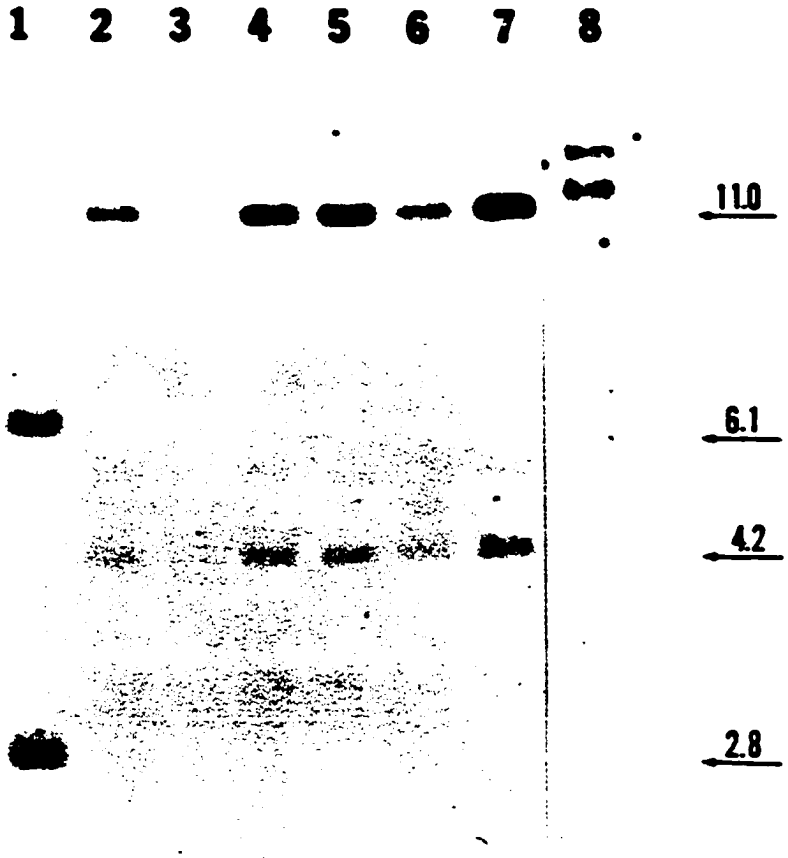
with these strains. However, the amount of a particular strain's individual plasmids vary because of the amount of the fragile large plasmids in each strain. Also, the size of the plasmids harboring nif genes varies greatly: from 112Mdal in strain PRC205 to 240 Mdal in strain PRC191. Therefore, it is significant that the plasmid pRjaPRC193 exists in all the fast-growing strains with such a large amount of sequence conservation.

7. Nif sequence conservation in slow-growing strains

It was of interest to obtain an estimate of the sequence conservation of nif genes in slow-growing strains. To answer this question pRmR2 DNA (containing nif DH genes from R. meliloti) was hybridized to a Southern blot of EcoR1 digested total DNA from nine different slow-growing strains. Six of the nine strains examined have identical hybridization patterns with relatively strong hybridization to a 11.0 kb fragment. A 4.2 kb fragment also hybridized as did two relatively weak signals of 3.3 and 5.4 kb, (Figure 5, lanes 2-7). A 1.8 kb band from strain 61A76 that hybridized is due to homology with the vector.

The plasmidless strain 3I1b110 had a different hybridization pattern with the nif probe in which two bands of 2.8 and 6.1 kb hybridized strongly and a 2.0 kb band hybridized relatively weakly (Figure 5, lane 1). The two other strains examined, AA102 and 3I1b123, both contain plasmids. The hybridization pattern of the nif

Figure 5: Hybridization of ^{32}P-labeled pRmR2 (containing *nif* DH from *R. meliloti*) to EcoRI-digested total DNA from slow-growing strains Lane 1, 3I1b110; lane 2, 3I1b94; lane 3, 3I1b74; lane 4, 3I1b143; lane 5, 3I1b71a; lane 6, 61A76; lane 7, 3I1b31; lane 8, AA102. Molecular weights are in kb and are explained in the text.


1
2
3
4
5
6
7
8
11.0
6.1
4.2
2.8

genes in each of these strains is distinctly different. Strain AA102 (Figure 5, lane 8) has two large bands of 12.0 and 13.7 kb that hybridize and strain 3I1b123 (results not shown) contains a 2.8-kb fragment, common to the same sized fragment in 3I1b110, and two other bands of 5.5 and 8.4 kb. Therefore, a great amount of conservation of nif gene sequences is found in the nine strains examined; although no definitive correlation may be made as to the size of a hybridizing nif fragment and the presence or absence of a plasmid(s) in the slow-growing strains.

8. Nif sequence conservation in fast-growing strains

The location of structural nif genes have previously been determined to be on large plasmids in the fast-growing strains, except for strain PRC194 (Masterson et al., 1982). The 76- and 145-Mdal plasmids in strain PRC194 did not contain structural nif genes. To estimate the sequence conservation of nif DNA in fast-growing R. japonicum strains, the Southern blot containing these plasmids (see Figure 2) was stripped of ^{32}P-probe and hybridized with ^{32}P-labeled pRmR2 DNA. The results are presented in Figure 6A. Every strain with structural nif genes on a large plasmid contained the same EcoR1 size fragments, 4.2 and 4.9 kb, which hybridized with the labeled nif probe. As expected, no hybridization was observed with the plasmid pRja61A76 from the slow-growing strain (lane 1 of Figure 6A) or with the plasmids isolated from fast-growing strain PRC194 (lane 2 of

Figure 6. Hybridization of ^{32}P-pRmR2 (containing *nif* DH from *R. meliloti*) to EcoRI digested plasmid DNA from fast-growing strains A: The Southern blot of the gel in Figure 2 was stripped of ^{32}P-probe (as described in the Materials and Methods section) and hybridized with the *nif* probe. Lane 1, pRja61A76; lane 2, pRjaPRC194a,b; lane 3, pRjaPRC201a,b; lane 4, pRjaPRC205a,b,c; lane 5, pRjaPRC206a,b,c; lane 6, pRjaPRC191a,b; lane 7, pRjaPRC193. B: ^{32}P-pRmR2 hybridized to EcoRI digested total DNA from fast-growing strains. The Southern blot of the gel in Figure 4 was stripped of ^{32}P-probe and hybridized with the *nif* probe. Molecular weights shown are the sizes in Kb of the EcoRI fragments that hybridize with the *nif* probe.

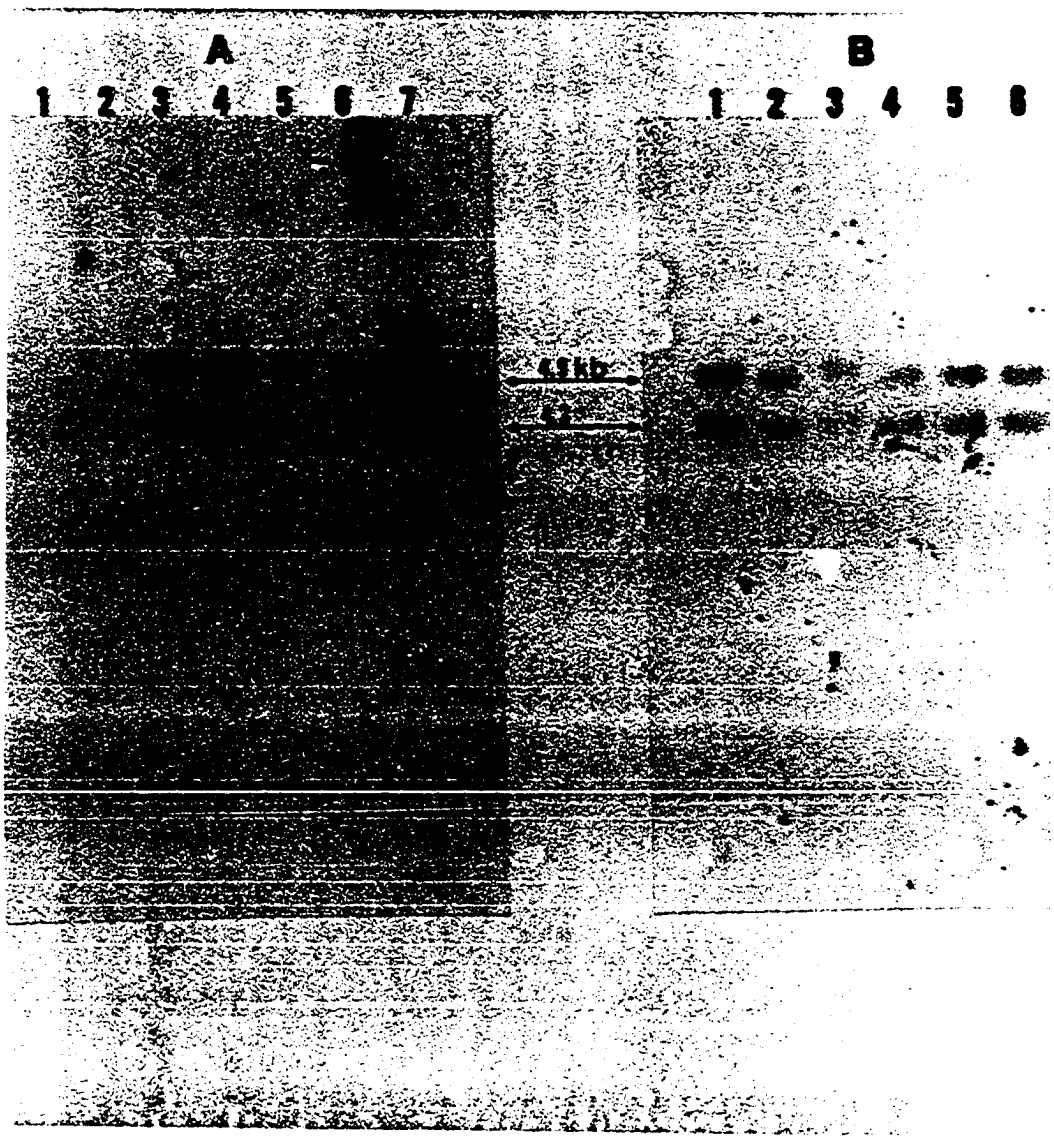
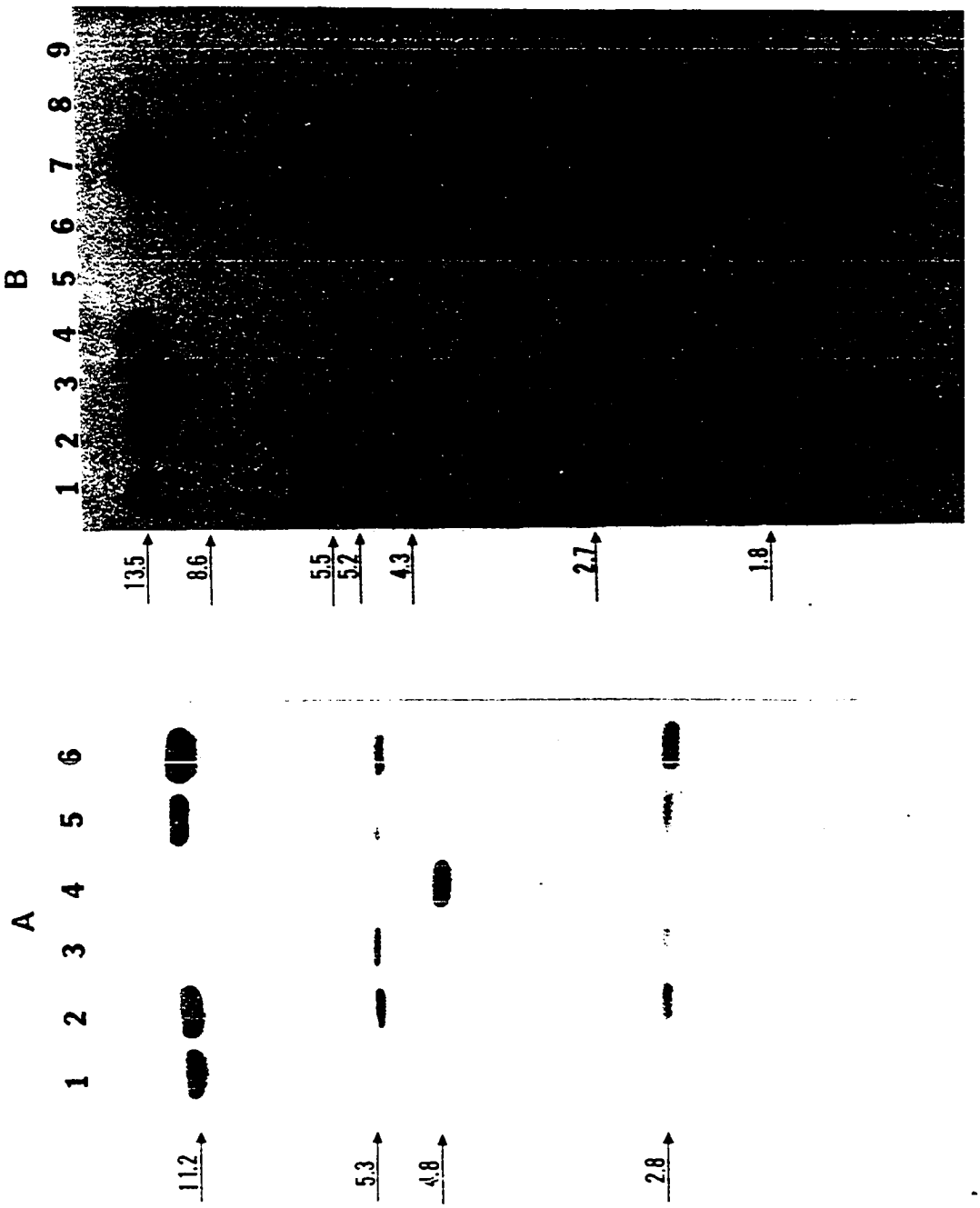

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6
4.8kb

Figure 6A). Furthermore, when the ^{32}P-labeled *nif* DNA was hybridized to total DNA from fast-growing strains (as in Figure 4), the same sized bands hybridized to strain PRC194 DNA (lane 6 of Figure 6B). These data indicate that *nif* structural genes are highly conserved even though their location may be on a 112-Mdal plasmid In strain PRC205, or plasmids larger than 190 Mdal in strains PRC191, PRC193, PRC201, and PRC206, as well on a megaplasmid or the chromosome in strain PRC194.

9. *Nod* sequence conservation in slow- and fast-growing strains

Using costramid clone pRmSL26 containing a nodulation gene from *R. meliloti* (Long et al., 1982) (the kind gift of S. Long and F. Ausubel, Harvard U.) allowed the further isolation of a 3.5 kb fragment known to carry essential *nod* genes (S. Long, Stanford U., personal communication). The *nod* probe was isolated by R. K. Prakash in this laboratory. When this *nod* fragment was hydridized to EcoR1-digested plasmid DNA from fast-growing strains, the result was that both common and unique bands of the large plasmids hybridized (Figure 7A). Lane 1 of Figure 7A contains an EcoRi digest of total DNA from strain PRC194. Although *nif* genes are conserved in this strain similarly to those of other fast-growing *R. japonicum* strains examined, the nodulation genes seem to have two bands in common (11.2 kb and 5.3 kb) with EcoR1 fragments of plasmids from strains PRC201, PRC191, and PRC193 (Figure 7A, lanes 2, 5, and 6). However, a smaller

Figure 7. Hybridization of ^{32}P-labeled *nod* genes to EcoRI digested plasmid DNA from fast-growing strains A: A 3.5 Kb EcoRI-BamHI fragment, isolated from a clone known to carry nod genes (described in the text) was hybridized to a Southern blot containing: lane 1, total DNA of PRC194; lane 2, pRjaPRC201a,b; lane 3, pRjaPRC205a,b,c; lane 4, pRjaPRC206a,b,c; lane 5, pRjaPRC191a,b; lane 6, pRjaPRC193. B: Hybridization of the same *nod* probe to EcoRI digested total DNA from slow-growing strains under reduced stringency conditions. Lane 1, 3I1b31; lane 2, 61A76; lane 3, 3I1b71a; lane 4, 3I1b143; lane 5, 3I1b74; lane 6, 3I1b94; lane 7, 3I1b110; lane 8, 3I1b123; and lane 9, AA102. Molecular weights shown are the sizes in kb which hybridize with the *nod* probe.


A
1 2 3 4 5 6
11.2
5.3
4.8
2.8
B
1 2 3 4 5 6 7 8 9
13.5
8.6
5.5
5.2
4.3
2.7
1.8

band (4.8 kb) is common between plasmids isolated from strains PRC194 and PRC206 (Figure 7A, lanes 1 and 4). This hybridization assay indicates that some diversity exists in the EcoR1 restriction enzyme sites of nodulation genes in fast-growing R. japonicum.

In contrast, the same 3.5-kb fragment hybridized very weakly to total DNA from slow-growing strains under the hybridization conditions used in this study. Under special, reduced hybridization conditions some hybridization is observed with the same probe. Figure 7B shows the result of hybridization with the nod probe and EcoRI digested total DNA from slow-growing strains. Three bands are predominnant in the hybridizations (13.5 kb, 4.3 kb and 2.7 kb). It is unknown at this time whether the bands which hybridized are true nod homologous sequences. The conservation of similar sized EcoRI bands does, however, indicate some type of common relatedness. These results suggest that significantly different DNA sequences are involved in the symbiotic event of nodulation by slow-growing R. japonicum.

10. Significance of DNA relatedness in R. japonicum

The goal of this study was to determine the amount of relatedness within and between the plasmid DNA of slow- and fast-growing strains of R. japonicum and to relate this information to the conservation of genes that have symbiotic importance. It is evident from the hybridization assays in this study that a considerable amount of intra-relatedness exists between plasmid DNA sequences within slow-

and fast-growing R. japonicum. The plasmid pRja61A76, from slow-growing R. japonicum strain 61A76, is highly conserved in strains from diverse ecological and geographical locations in the United States and India. Strains 61A76 and 3I1b31 are in serogroup c3, and strains 3I1b71a and 3I1b143 are in serogroups c2 and 110-122, respectively (Table 1). Each of the other slow-growing R. japonicum strains examined fall into different serogroups; however, antibody from strains 3I1b110 and 3I1b143 do cross-react with each other (Keyser and Weber, 1979). Consequently, no overall correlation seemingly exists between the plasmid content of a strain and its serological group.

The classification of R. japonicum strains by solution hybridization of total DNA (Hollis et al. 1981) designates 3I1b31, 61A76, and 3I1b94 to the same DNA homology group. From our data, it would seem that strains 3I1b31 and 61A76 are very closely related. However, strain 3I1b94, with a different plasmid profile, must have a related chromosomal background. The hybridizations of Hollis et al. (1981) measured the amount of homology between DNA based on solution hybridizations of DNA isolated from a variety of R. japonicum strains. The hybridizations were performed at two different temperatures in order to vary the stringency of the hybridization conditions. Although this type of hybridization has value, especially in the number of hybridizations that can be performed simultaneously, it does not allow four important pieces of information revealed by Southern

hybridizations. First, the restriction digested and immobilized DNA on the nitrocellulose filters allow the determination of exactly which bands of DNA are homologous to the labeled DNA probe. Second, there is a choice as to the type of DNA on the filter as well as the DNA used as a labeled probe. That is, plasmid DNA may be immobilized on the filter and hybridized with labeled plasmid DNA. The solution hybridizations performed by Hollis et al. (1981) were done with total DNA which includes both plasmid and chromosomal DNA. If the plasmids of two strains were identical but the chromosomal background was different it would not be determined by the solution hybridization technique. Third, the location of DNA sequences may be determined by hybridizing labeled probes to hybridization filters which contain plasmid DNA, total DNA, or both types individually. Fourth, the use of Genescreen hybridization membrane (New England Nuclear) in this study has allowed the removal of labeled probe such that the immobilized DNA on the filter could be rehybridized.

The hybridization conditions employed in this study were relatively stringent and non-homologous hybridization was not observed. Lambda bacteriophage DNA was included as a negative control on all hybridization filters and occasionally plasmid pRja61A76 as either a negative or a positive control. Furthermore, the same hybridization results were obtained as those by Haugland and Verma (1981), who used a different Southern hybridization procedure, in that the slow-growing strain 3I1b110, which has no readily isolatable

plasmid, contains sequences homologous to pRja61A76.

The slow-growing strain 3I1b123, which does contain plasmid DNA (although non-homologous to pRja61A76), also contains sequences in its genome that cross-hybridize with the plasmid pRja61A76. Inasmuch as this plasmid is highly conserved it is possible that information on the plasmid may be of significant value. Berry and Atherly (1984) recently have described genome rearrangements prompted by R-group plasmids. Perhaps plasmid DNA in R. japonicum undergoes excision and integration with its host chromosome and may explain the presence of plasmid sequences in the chromosome or megaplasmid of strains 3I1b110 and PRC194.

The plasmid pRjaPRC193, isolated from fast-growing R. japonicum strain PRC193, also is highly conserved in the fast-growing strains. Analogous to the finding of pRja61A76 sequences in plasmidless slow-growing strain 3I1b110, the plasmid sequences of pRjaPRC193 are in all fast-growing strains as well as in strain PRC194, which does not contain symbiosis genes on its plasmids. Strain PRC194 contains plasmids with little or no homology with pRjaPRC193. Therefore, it seems that the plasmid pRjaPRC193 sequences are integrated into strain PRC194 DNA and are present as an essential ingredient of all R. japonicum fast-growing strains. It is clear that plasmid pRjaPRC193 is highly conserved in the fast-growing strains isolated from diverse provinces in the People's Republic of China.

DNA sequence homology studies, using the Southern hybridization

technique, by Jouanin et al. (1981) have shown different digest patterns and conserved regions of homology in the plasmids of R. meliloti, which is similar to the results that we have presented for the plasmids of fast-growing R. japonicum in this study. However, the conserved sequences did not contain regions with homology to nitrogen fixation probes. Hybridization with a nodulation probe was not available and consequently not used in this particular study. Therefore, the work of Jouanin et al. (1981) demonstrates conservation of sequences which may or may not be involved in the symbiosis process. Other DNA homology studies (Hollis et al., 1981; Jarvis et al., 1983) show wide variation in the relatedness of Rhizobia. Hollis et al. (1981) separated the slow-growing R. japonicum into three separate homology groups based on solution hybridizations. Jarvis et al. (1983) showed variable conservation between different Rhizobium species based on hybridizations with cloned nodulation sequences.

Homology between plasmids isolated from A. tumefaciens, a bacterium of the same family as Rhizobium and responsible for tumor induction (Ti) in dicotyledenous plants, have revealed highly conserved regions in a large number of different strains (Drummond and Chilton, 1978; Knauf et al., 1983; and Thomashow et al., 1981). Costantino et al. (1981) has demonstrated a great deal of conservation in the plasmids of another species of Agrobacterium, that being A. rhizogenes. Relatedness between wide host range octopine and nopaline Ti plasmids is 6-15% whereas the homology between limited host range

octopine Ti plasmids is approximately 64% (Thomashow et al., 1981). The same may be true for large plasmids in R. japonicum and other Rhizobia species which contain host-specificity genes responsible for recognition of a specific host.

Structural nif genes are strongly conserved in all nitrogen-fixing organisms (Ruvkun and Ausubel, 1980). The nif genes in slow-growing strain 3I1b110 have been cloned and characterized by Hennecke and others (Fuhrmann and Hennecke, 1982; Hennecke, 1981; and Kaluza et al., 1983). In this study, hybridization using a nifDH probe from a R. meliloti strain has shown strong organizational conservation in six of the nine slow-growing strains examined. The organization of nif in these strains does not seem to be related to the presence or absence of a particular plasmid. Hybridization with the same nif probe to fast-growing strains showed an identical hybridization pattern to plasmids harboring nif DNA as well as to strain PRC194, which contains nif genes on a megaplasmid or the chromosome. Hybridization with individual nif fragments has revealed that two copies of nif structural genes may be present in the fast-growing R. japonicum (J. Noti, Department of Plant Biology, Boyce Thompson Institute, personal communication). Reiteration of nif genes has been reported in R. phaseoli by Quinto et al. (1982) and is therefore not a totally unexpected phenomena.

Nodulation gene sequences do not seem to be extensively related between slow- and fast-growing R. japonicum strains. A 3.5-kb nod

probe, originally isolated from R. meliloti costramid pRmSL26 (Long et al., 1982) and known to carry essential nodulation genes (S. Long, Department of Genetics, Stanford U., personal communication) hybridized to several EcoRI fragments of plasmid DNA isolated from fast-growing R. japonicum strains. A clone carrying the 11.2-kb fragment was transformed into Agrobacterium tumefaciens, which has resulted in the formation of nodules (R. K. Prakash and A. G. Atherly, Department of Genetics, Iowa State University, personal communication). Site-directed mutagenesis (Ruvkun and Ausubel, 1981) is now being performed to determine the exact location of the nodulation genes. The other bands that have hybridized with the nod probe also are being investigated. However, the hybridization of this probe to the total DNA of slow-growing R. japonicum yielded results only under reduced stringency conditions and the bands which hybridized may or may not be authentic nod sequences. That such differences exist between nodulation genes of the slow- and fast-growing strains indicates a possible evolutionary divergence in the nodulation properties of these bacterial symbionts.

In conclusion, the genetic content of slow- and fast-growing strains varies with respect to the organization and location of symbiotic and nitrogen-fixation genes and their amount of relatedness. The ability of both types of strains to nodulate a single soybean cultivar (Keyser et al., 1982) and yet hybridize under different conditions to a common nodulation probe, indicates the presence of

other nodulation genes. The nodulation genes of both slow- and fast-growing R. japonicum now are being examined in detail in order to determine the genetic basis of nodulation in the bacterial symbiont of the soybean plant.

III. COSMID CLONING AND MAPPING A LARGE *RHIZOBIUM JAPONICUM* PLASMID

A. Introduction

In the last section, the plasmid pRjaPRC193 from fast-growing strain PRC193 was used as a hybridization probe and also to determine the plasmid location of structural *nif* and *nod* sequences. Interesting phenomena including reiteration of symbiotic genes and the presence of the plasmid in the chromosome of another strain were observed. Of all the fast growing strains examined it was only PRC193 which contained a single, 350 kb plasmid. The examination and use of a single plasmid eliminated problems associated with the separation of multiple plasmids within a given fast-growing strain. Therefore, a physical and genetic map of pRjaPRC193 seemed an important aspect of the laboratory goal of understanding *R. japonicum* at the molecular level.

Only a few examples exist of physical and genetic maps in *Rhizobium*. Prakash et al. (1982) presented evidence for a restriction map of pRle1001a, a 225 Kb plasmid harboring symbiotic genes, from *R. leguminosarum*. The 225 Kb non-symbiotic plasmid pRme41a of *R. meliloti* was physically mapped with restriction enzymes by Banfalvi et al. (1981). Another study physically and genetically mapped a 135 Kb region of pRme41b, a megaplasmid containing symbiotic genes in *R. meliloti* (Kondorosi et al. 1984). Other mapping studies of *Rhizobium*

have concentrated on specific regions containing genes involved with symbiosis and have ignored the sequences surrounding these areas (Long et al., 1982; Schofield et al., 1983).

The most significant obstacle encountered in mapping a Rhizobium plasmid is their large size. Simple plasmid mapping schemes have been used to map many different types of DNA (Smith and Birnstiel, 1976). These schemes involve cloning 15 Kb or less insert molecules into a small vector such as pBR322. The plasmids pRle1001a and pRme41a mentioned above were mapped in this way with the aid of Southern hybridizations. However, for plasmids larger than 100 kb, this type of simple mapping is tedious and time consuming. Computer programs are now available for restriction mapping and are especially valuable with small clones, but are limited as to the size of DNA being mapped (Nolan et al., 1984). Important advances using Lambda bacteriophage as a cloning vehicle, resulted in methods whereby up to 46 Kb of insert DNA could be 'packaged' in a single clone. This method is called cosmid cloning and was used in this study (Collins and Hohn, 1978). The cosmid vector used was pVK102 which contained 21 Kb inserts of HindIII digested pRjaPRC193.

The other major obstacle met in this study was the occurrence of many repeated sequences. Not only were the symbiotic genes reiterated, but other sequences were also repeated throughout the plasmid pRjaPRC193. This meant that Southern hybridizations, including a new technique, Southern cross restriction mapping, were difficult to

interpret due to extra bands hybridizing. Walking around the plasmid using cosmid clones as probes was severely restricted by this important factor. However, valuable information was obtained and contributed to the map of large regions of pRjaPRC193.

Over 90% of the plasmid has been mapped with HindIII, HpaI and KpnI, including a single stretch over 240 Kb long containing the symbiosis genes. As of the writing of this thesis, the regions of the plasmid pRjaPRC193 mapped in this study represent the largest physical and genetic map of any plasmid in Rhizobium or, as far as can be determined, any strain of bacteria. A complete circular map is still a goal of this laboratory and is being pursued at this time.

B. Materials and Methods

1. Bacterial strains and media

E. coli K12 strain HB101 (Boyer and Roulland-Dussoix, 1969) and E. coli lambda bacteriophage packaging strains NS428 and NS433 (Sternberg et al., 1977) were obtained from David Morris, Dept. of Genetics, Iowa State U. E. coli K12 strain HB101(pVK102) was used as the source of the broad host-range cloning vector pVK102 (Knauf and Nester, 1982) and was obtained from Vic Knauf, University of Washington, Seattle, WA.

E. coli strains were grown in standard LB medium which contained per liter: 10 g Bacto-tryptone (Difco); 5 g yeast extract; and 10 g

NaCl. LB medium used in transduction assays contained 0.4% maltose.

2. Plasmid isolation and gel electrophoresis

Isolation of intact plasmid DNA from R. japonicum and E. coli were done according to the methods described in Section II. Agarose gel electrophoresis conditions were also the same as described in Section II except, however, different conditions were used for gels in the Southern Cross assays. In these assays, electrophorsis was performed using 0.7% agarose gels (Seakem) 5mm thick and 20 by 25 cm in size. A single comb, 18.5 cm wide, 0.1 cm thick, was used to cast a large trough for the application of DNA. Usually 4-5 ug of digested plasmid DNA, suspended in 300 ul of low TE (10 mM Tris (pH 8.0), and 1 mM EDTA) and 30 ul of tracking dye (50% Ficol, 0.25% bromophenol blue), was carefully layered into the well. Optimum electrophoresis conditions were 2 V/cm for 18 h at room temperature. Under these conditions, digested DNA fragments were separated as sharp, linear bands, which were subsequently stained with ethidium bromide (0.5% ug/ml) and photographed as described in Section II.

3. Cosmid cloning

The in vitro packaging method of Sternberg et al. (1977), with modifications made by David Morris, Iowa State U., was used in the cosmid cloning of pRjaPRC193. An overnight culture of E. coli strain NS428 was examined for contaminants and 1.5 ml was added to 150 ml of

ml of LB broth. Similarly, 7.5 ml of strain NS433 was added to 750 ml of LB broth. Both cultures were subjected to vigorous shaking at 32° C until an $O.D._{630}$ of 0.2 was attained for each culture. Each culture was brought to 45° C in hot water in order to induce the lysogens and were then placed at 37° C for an additional 90 minutes with vigorous shaking. After 60 minutes of shaking at 37° C, one ml samples were removed and tested for lysogeny with chloroform. At the end of 90 minutes, each flask was rapidly cooled in an ice-water bath.

A mixture of 150 ml each of the cold strains NS428 and NS433 were centrifuged at 8,000 rpm, 4° C, in a Sorvall centrifuge for 10 minutes. The mixed pellet was drained and resuspended in 0.6 ml of cold buffer A (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM $MgCl_2$, and 5 mM beta-mercaptoethanol). The mixture was transferred to a 1.5 ml polypropylene tube on ice in preparation for sonification (Heat Systems-Ultrasonics, Inc.). Ethanol was used to sterilize the tip of a sonicator and the mixture was subjected to 5-6 two second periods of sonification with an output corresponding to approximately 70 watts. After each 2 second period, the sample was allowed to cool and the amount of viscosity was measured by drawing small samples into an eppendorf pipette tip and releasing it back into the 1.5 ml tube. At the end of the fifth sonification, the sample had lost a great amount of viscosity but could still form droplets at the tip of an eppendorf pipette tip. One sixth ml of cold, sterile gycerol was added to the tube and gently mixed with an eppendorf pipette tip. Sterile 1.5 ml

polypropylene tubes were placed on ice and 50 ul of sample were added to each tube and rapidly immersed in liquid nitrogen. The tubes were labeled as 'extract A' and then stored at -70^{o} C until needed.

The remainder of the NS433 culture was centrifuged in the cold at 8,000 rpm for ten minutes in a Sorvall centrifuge. The pellet was gently resuspended in 1.2 ml of Tris-sucrose buffer (50 mM Tris-HCl (pH 7.4), and 10% sucrose). Polypropylene tubes were placed on ice and 80 ul of the mixture was added to each tube and then rapidly frozen in liquid nitrogen. Half of the tubes were quickly thawed in room temperature water, followed by another cycle of freezing and thawing. Each tube then received 4 ul of a 1 mg/ml lysozyme solution (lysozyme in 0.25 M Tris-HCl, pH 8.0) and were gently stirred with an eppendorf pipette tip. The mixtures were placed on ice for 30 minutes. During this time, the other half of the tubes were thawed and the same protocol was followed. A mixture of 0.625 ml cold glycerol and 0.2 ml of buffer B (6 mM Tris-HCl (pH 7.4), 15 mM ATP, 16 mM $MgCl_2$, 60 mM spermidine-HCl, 100 mM putracine, and 30 mM beta-mercaptoethanol) were prepared and 33 ul was added to each tube and gently mixed. These tubes were labeled as 'extract B' and then placed at -70^{o} C until needed in the packaging reaction. The time periods in the preparation of the packaging mixtures require that the entire process be started early in the day in order to ensure completion of both extract mixtures.

In order to prepare the plasmid-vector DNA for the packaging

mixtures, a complete digest of the vector and a partial digest of the plasmid had to be completed. The 23 Kb cosmid vector pVK102 has two regions with antibiotic markers for inactivation and/or selection, namely, kanamycin and tetracycline. The restriction enzyme HindIII recognizes a single site in the kanamycin region of pVK102. All inserts of foreign DNA in this site render cells with these recombinants sensitivity to kanamycin. One ug of pVK102 was digested to completion with 10 u of HindIII using the conditions described for restriction enzyme digestions in Section II. A partial digest of pRjaPRC193 was achieved by the addition of 10 u of HindIII to 1 ug of insert followed by serial dilution to tubes containing 1 ug of plasmid each such that a final concentration of less than 0.125 u of HindIII in the final tube was achieved. A relatively thin (2 mm thick) gel was prepared at a low 0.4% concentration in order for maximum separation and economical use of the partially digested plasmid DNA. Only 0.1 ug of each partial digest was required in each lane of the gel for determination of digestion. Samples which had the brightest intensity of fluorescence in the 20-30 Kb region, when stained with ethidium bromide and visualized with ultraviolet light, were selected as insert DNA for ligation with pVK102.

A 3:1 ratio of insert to vector DNA, at a final concentration of 0.3 ug/ul, was chosen for ligation. Partially digested pRjaPRC193 and completely digested pVK102 were mixed together and ethanol precipitated. The mixture was resuspended in 8 ul of TM buffer (10 mM

Tris-HCl (pH 8.0), and 10 mM $MgCl_2$) and placed at 65° C for 5 minutes followed by a slow (3-4 h) cooling period to room temperature. The sample was placed on ice and 1 ul of ligation buffer (100 mM Tris-HCl (pH 7.4), 100 mM dithiothreitol, and 50 mM ATP (pH 7.0)) and 1 ul of ligase (New England Biolabs) were added and gently mixed together. The mixture was placed at 14° C overnight to allow ligation of insert and vector DNA. Another thin, low percentage, gel was prepared and 1 ul of the ligation mixture was applied and separated in order to determine the success of ligation. Control standards included intact, EcoRI digested, and HindIII digested lambda bacteriophage DNA. The ligated mixture resulted in fragments approximately 49 Kb in size, which indicated ligation had occurred.

The packaging reaction occurred in the following order: at room temperature 30 ul of buffer A, 2 ul of buffer B, 20 ul of extract A, and 5 ul of ligated DNA, were gently mixed together and incubated at room temperature for 15 minutes. A frozen tube containing extract B was allowed to thaw while the above mixture was stirred in with a sealed, glass capillary tube. The entire mixture was placed at 37° C for 60 minutes. After this incubation, 150 ul of DNAse solution (1 mg/ml DNAse in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 10 mM $MgSO_4$) were added, gently mixed, and allowed to incubate another 15 minutes at 37° C. 100 ul of chloroform was added and the tube was placed in an eppendorf centrifuge and spun for 2 minutes. The supernatant, containing phage, was placed at 4° C until needed.

An overnight culture of E. coli strain HB101 was diluted 1:50 in fresh LB media, supplemented with 0.4% maltose, and was allowed to reach late log growth. The cells were centrifuged in the cold for 10 minutes at 7,000 rpm followed by resuspension in 0.1 volume 10 mM $MgCl_2$. In a 1.5 ml eppendorf centrifuge tube, 0.2 ml of the cells was mixed with 0.1 ml 10 mM $MgCl_2$ and 50 ul of phage, and placed at room temperature for 15 minutes to allow phage absorption. One ml of LB media were added and the tube was transferred to a 37^{o} incubator for 60 minutes to allow expression of antibiotic resistance genes. Three ml of a soft, 0.8% Noble agar (Difco) overlay containing 10 ug/ml of tetracycline were added to the cells and immediately poured over 15 ug/ml tetracycline LB agar plates. Resulting Tc resistant colonies were examined for inserts by growth on 50 ug/ml kanamycin LB agar plates.

4. Southern cross restriction mapping

A packaged kit was purchased which included chemicals and instructions for this type of mapping (New England Nuclear). Large regions of pRjaPRC193 were mapped and repetitive sequences were determined using HpaI and HindIII digests of pRjaPRC193.

The first step in the method involves the preparation of 'hot' and 'cold' Southern transfers. Agarose gels were prepared according to the procedure described earlier for gel electrophoresis. Only one gel contained ^{32}P end-labeled plasmid, while the other gels contained

non-labeled pRjaPRC193. Southern transfers were done exactly as described before. The hot membrane transfer in this case was 2 ug of HindIII digested pRjaPRC193, while the cold transfers were 2 ug each of HindIII and HpaI digested pRjaPRC193. The cold transfers were prepared first and used GeneScreen Plus transfer membrane. The plasmid DNA was allowed to bind to the membranes by drying overnight at room temperature. After the cold transfers were completed, HindIII digested pRjaPRC193 was end labeled by adding the following chemicals sequentially: 1 u of DNA polymerase I (Klenow fragment) and 2 ul each of 'hot' dCTP and dATP (final concentration 2 mM each, specific activity of 600 Ci/mM, labeled with ^{32}P in the alpha position; New England Nuclear) and 2 ul each of 'cold' dTTP and dGTP (final concentration 2 mM each). The sample was mixed and incubated for 30 minutes at 37^{o} C. Two ethanol precipitations were done to remove unincorporated nucleotides and the DNA was resuspended in 300 ul low TE and 30 ul tracking dye as previously described in this Section.

Agarose gel electrophoresis was done in exactly the same manner as the previous 'cold' transfers except that a ventilated hood was used for both electrophoresis and the Southern transfer of the 'hot' digest. Also, the transfer of the ^{32}P-labeled DNA was to regular GeneScreen transfer membrane (New England Nuclear). The purpose of using this membrane is that the 'hot' transfer would <u>not</u> bind; 2 h at 80^{o} C is required to fix ssDNA to this type of membrane. During the last few hours of the 'hot' transfer, the 'cold' membranes were

prepared for hybridization in the following manner. The HindIII and HpaI blots were prewashed for 3 hours in hybridization buffer A (100 ml hybridization buffer concentrate, 100 ml H_2O, and 2 ml 10% SDS). The HpaI membrane, to be placed adjacent to the 'hot' HindIII transfer, was prewashed separately in hybridization buffer B (25 ml hybridization buffer concentrate, 25 ml H_2O, 0.5 ml 10% SDS, and 0.5 ml sodium pyrophosphate) for 10 minutes. The 'cold' HindIII membrane was washed for 10 minutes in hybridization buffer C (125 ml hybridization concentrate, 125 ml formamide, and 2.5 ml 10% SDS).

After the labeled DNA was transferred, the regular GeneScreen membrane was removed from the Southern blot apparatus and rinsed for one minute in degassed 25 mM sodium phosphate buffer, pH 6.8. The 'hot' transfer membrane was placed on a clean, glass plate DNA side up and 'north' with respect to the position of the largest band on the membrane. The HpaI membrane was placed DNA side down and the largest DNA band was positioned 'east' on top of the 'hot' HindIII membrane. The 'cold' HindIII membrane was placed on top of the HpaI membrane in the same orientation as the 'cold' HpaI transfer membranes. A piece of Whattman 3M paper, the same size as the hybridization membranes and soaked in hybridization buffer C, was placed on top of the membranes. Eight ml of hybridization buffer C was poured over the surface of the membranes and paper, and a glass plate was placed on top and secured on four sides with large, strong clamps. The apparatus was placed in a 40^{o} C incubator overnight. Therefore, the two 'cold' membranes were

DNA side down and positioned 90^{o} opposed to the largest DNA band on the 'hot' membrane. Since the ^{32}P-labeled DNA was not bound by baking to the GeneScreen membrane, it was free to diffuse up and through the permeable, GeneScreen Plus membranes. Under these hybridization conditions, homologous DNA sequences formed stable, double-stranded hybrids.

The following day, the apparatus was disassembled, the membranes were placed in a 20 by 30 cm Seal-A-Meal bag (Sears), and 100 ml of wash solution A (2X SSC, 0.1% SDS) were added and gently rocked on a platform shaker at room temperature for 20 minutes. This was repeated two times followed by 2-40 minute washes in wash solution B (0.1X SSC, 0.1% SDS) at 50^{o} C. After the membranes had been washed to remove non-homologous labeled DNA, each membrane was exposed to X-ray film as previously described in Section II.

5. Southern blots and hybridizations

The same conditions described in Section II for Southern blots and hybridizations were used in the examination of cloned pRjaPRC193 inserts with various hybridization probes. Lambda bacteriophage DNA served as both a negative control and molecular weight markers on all transfer membranes.

C. Results and Discussion

1. Cloning strategy

The preparation of a special clone bank of pRjaPRC193 was required for physical and genetic mapping. This section is about the problems associated with mapping a very large piece of DNA and the subsequent cloning strategy used. Two major problems were encountered in mapping the large, 350 Kb Rhizobium plasmid. First, the size of the plasmid is 100X larger than most cloning vehicles; the common vector pBR322 is a mere 4.3 Kb (Sutcliffe, 1979). Most cloning and mapping strategies are designed for DNA less than 50 Kb. In order to produce a clone bank representative of all plasmid sequences, a cosmid clone bank was made in a wide host range vector. Second, many repeated DNA sequences are distributed throughout the plasmid which hinder experiments dependent on DNA:DNA hybridizations, such as Southern hybridizations.

The physical mapping by molecular cloning presents a number of complications due to the plasmid's large size. To represent the entire plasmid for P=0.99 in a clone bank of pBR322, for instance, would require 158 transformants with an average insert size of 10 Kb. This uses the relationship:

$$\ln (1 - 0.99)/\ln(1 - (X/Y)),$$

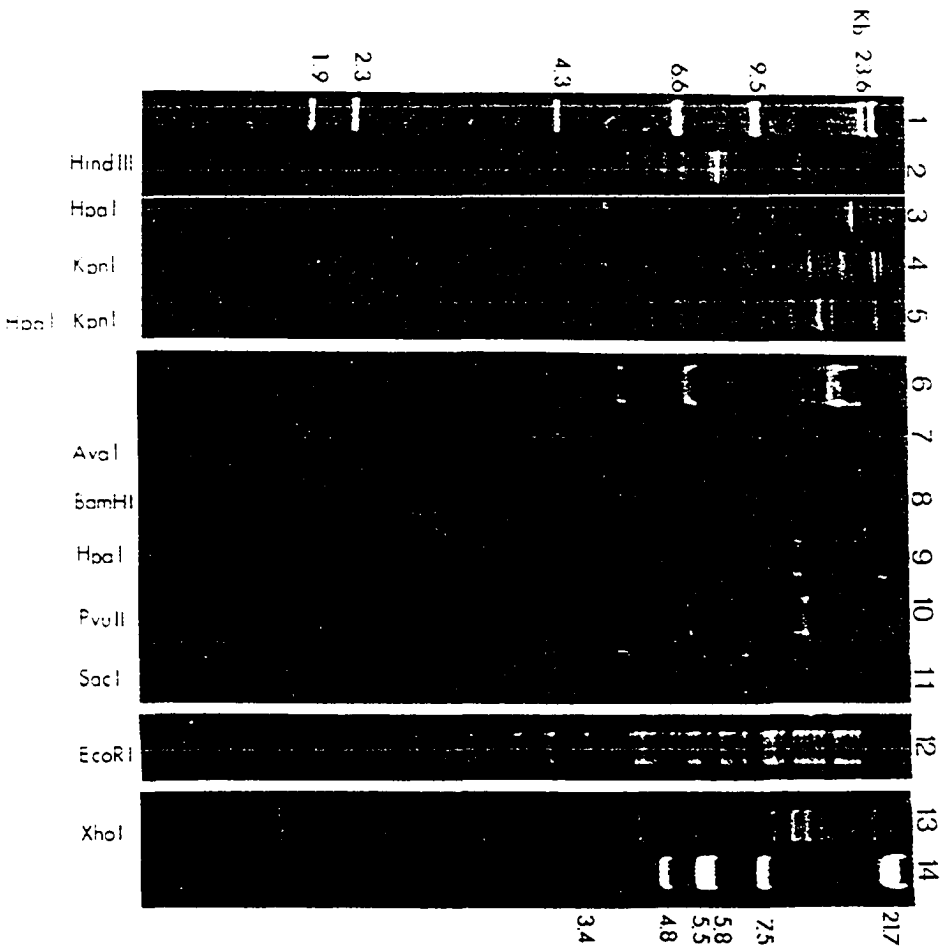
where X is the size of inserts (Kb) and Y is the size of the molecule being cloned (Kb). However, even assuming favorable conditions, which

includes overlapping sequences and a high transformation frequency, the number of clones is too large for a single person to examine. The limiting factors include: the number of agarose gels used, the space per gel (each gel has space for 20 different samples), and the time it takes for a single run, which is usually 18 h. A given clone is usually examined with several restriction enzymes such that single, double, and even triple digests are done. With molecular weight standards included, only 6-7 clones can efficiently be examined on a gel. The selection of DNA for gels is based on the premise that one knows the content of a given clone, patterns of overlaps, and that each clone contains unique DNA sequences. This in itself is a time consuming process.

Another limiting factor is the amount of 'walking', using clones as hybridization probes in the Southern hybridization method, required to examine a piece of DNA 350 Kb long. Assuming the average clone is 10 Kb long, at least 35 hybridizations would be needed. A single hybridization, starting with digesting selected clones and ending with an autoradiograph, takes approximately 6 days under optimum conditions. An additional problem is the plasmid pRjaPRC193 contains many repeated sequences which obscure hybridization results. This unique problem will be examined in future sections.

An important consideration in any mapping-cloning strategy is the cloning vector itself. Ideally, a vector is relatively small, has single restriction sites, and has at least one selectable marker.

Figure 1. Agarose gel electrophoresis of plasmid pRjaPRC193 digested with a variety of restriction endonucleases Lane 1, HindIII digest of Lambda bacteriophage DNA; lane 2, HindIII digest; lane 3, HpaI digest; lane 4, KpnI digest; lane 5, HpaI/KpnI double digest; lane 6, same as lane 1; lane 7, AvaI digest; lane 8 BamHI digest; lane 9, HpaI digest; lane 10, PvuII digest; lane 11, SacI; lane 12, EcoRI digest; lane 13, XhoI digest; and lane 14, Lambda bacteriophage digested with EcoRI. Lambda DNA was used as molecular weight standards, shown in kb, and as negative controls in Southern hybridizations.


Kb 23.6
9.5
6.6
4.3
2.3
1.9
HindIII
HpaI
KpnI
HpaI KpnI
AvaI
BamHI
HpaI
PvuII
SacI
EcoRI
XhoI
1 2 3 4 5 6 7 8 9 10 11 12 13 14
21.7
7.5
5.8
5.5
4.8
3.4

Continuing with pBR322 as an example, cloning the plasmid pRjaPRC193 with this vector would pose special problems. The only enzymes which provide insertional inactivation of a selectable marker, in this case BamHI, HindIII, and SalI for tetracycline resistance, and PstI for ampicillin resistance, are not suitable with pRjaPRC193. Two of the largest bands of pRjaPRC193 digested with HindIII are 16 and 18.5 Kb in size (Figure 1). Digests with BamHI or SalI contain several large bands between 15 and 20 Kb in size (data not presented). PstI was not used in this study. The limits of efficient transformation in E. coli are about 15 Kb (Cohen et al. 1972). Thus, transformants including the largest restriction fragments of pRjaPRC193 digested with BamHI, HindIII, or Sal I, and the vector pBR322, approach 25 Kb in size and would be rare or nonexistent entities in a given transformation. This problem is more acute with other cloning vectors which have HpaI, KpnI, and XbaI sites. The largest bands of pRjaPRC193 digested with these enzymes are: 49 Kb, HpaI; 49 Kb, KpnI; and approximately 70 Kb, XbaI (Figure 1).

In order to circumvent the cloning problems associated with pBR322, a cosmid clone bank was made with the wide host range vector pVK102. Cosmid cloning is a procedure which involves the substitution of insert and vector DNA for lambda bacteriophage DNA in an in vitro packaging system (Collins and Hohn, 1978). The DNA sequence required by lambda phage proteins in the packaging system is lambda cohesive end sites, known as the cos sequence. In most cosmid vectors,

including pVK102 used in this study, the cos region is only 1.7 Kb long. The packaging capacity of the lambda bacteriophage head is rather large: 46-49 Kb. Therefore, very large inserts, containing vectors with the cos site, can be packaged and subsequently used to infect E. coli for examination of content.

In this study, the cosmid pVK102 was chosen for several reasons. First, the wide host range capabilities of the vector allow replication in both E. coli and Rhizobium. Cloned inserts of the plasmid can be shuttled back and forth between the two systems. Second, two sites for insertional inactivation are located on the cosmid, which include HindIII and XhoI in the kanamycin region, and SalI in the tetracycline gene. Third, the size of the cosmid is 23 Kb in size. While this only allows inserts of about 26 Kb, the vector is larger than digests of insert DNA and, with a slower mobility in gels, does not migrate with insert DNA. The large size of the cosmid is due to a 19 Kb EcoRI-BglII fragment derived from pRK290, a broad host range plasmid (Ditta et al. 1980). Apparently, a significant amount of pRK290 must be retained for broad host range capability (Knauf and Nester, 1982). An estimate of the number of clones needed to have 99% of the plasmid sequences represented in a clone bank, using the formula shown above, revealed that only 60 clones were required. Therefore, a cosmid clone bank using pVK102 and pRjaPRC193 was prepared.

The enzyme HindIII was chosen as it digests pRjaPRC193 into 60 or

Table 1. Molecular weights of pRjaPRC193 enzyme digests

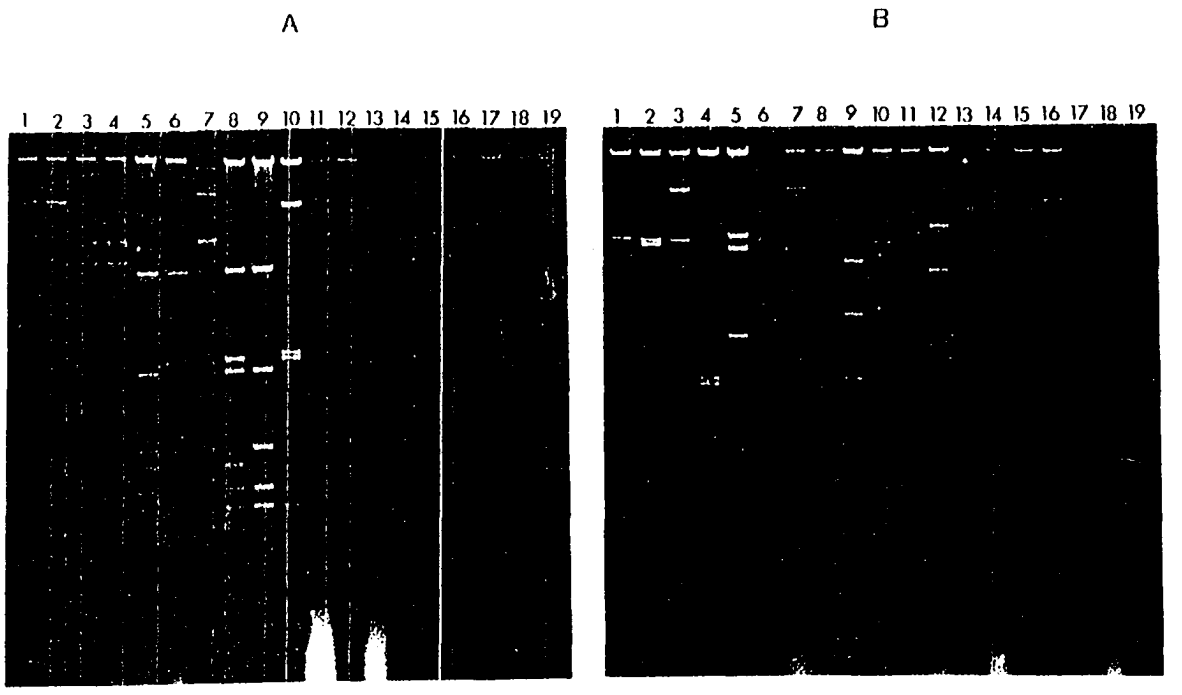
HindIII		HpaI		KpnI	
1.	18.5 Kb	1a,b	49.0 Kb	1.	49.0 Kb
2.	16.0	2.	32.0	2a,b	32.0
3.	13.7	3.	24.5	3a,b	27.4
4.	12.7	4.	23.1	4.	19.0
5a,b	12.0	5a,b	20.0	5a,b,c	17.8
6.	10.7	6.	18.5	6.	15.5
7.	10.5	7.	16.5	7.	14.5
8.	9.8	8.	15.5	8.	13.6
9.	8.8	9.	11.5	9a,b	12.5
10a,b	8.3	10.	11.0	10.	12.0
11a,b,c	8.0	11a,b	10.0	11.	9.8
12a,b	7.6	12.	9.8	12.	8.7
13.	7.5	13.	8.8	13.	8.3
14.	7.0	14.	8.2	14.	6.3
15a,b	6.8	15.	6.0	15.	4.5
16a,b	6.4	16.	5.2	16.	4.3
17.	6.0	17.	3.5	17.	3.9
18a,b	5.6	18.	3.4	18.	2.6
19a,b	5.2	19.	3.1	19a,b	2.3
20a,b	5.0	20.	2.6	20.	1.4
21a,b,c	4.4	21.	1.1	21.	0.8
22.	4.2				
23.	4.0	Total:	362.3 Kb	Total:	376.0 Kb
24a,b,c	3.8				
25.	3.6				
26.	3.5				
27.	3.4				
28.	3.3				
29.	3.2				
30.	3.0				
31a,b	2.9				
32a,b	2.7				
33.	2.6				
34a,b	2.4				
35.	2.3				
36a,b	2.2				
37.	2.1				
38.	2.0				
39.	1.9				
40a,b	1.8				
41.	1.7				
42.	1.6				
43.	1.5				
44.	1.3				
Total:	350.9 Kb				

more fragments; forty-four of the largest fragments range from 18.5 to 1.3 Kb (Table 1). Cosmid cloning was done according to the method of Sternberg et al. (1977) and is described in the Materials and Methods section. One hundred eleven tetracycline resistant clones were tested for kanamycin sensitivity to determine the presence of recombinant clones. Clones which were only tetracycline resistant were examined for plasmid content. Plasmids were isolated by the method of Holmes and Quigley (1981), and examined on agarose gels as described in the Materials and Methods section.

2. Characterization of cosmid clones

Figure 2 contains two representative gels of cosmid DNA digested with HindIII. The largest band in each lane is the 23 Kb vector pVK102. Small amounts of pRjaPRC193 were applied to two lanes in each gel as molecular weight standards and for positive hybridization controls. Since many HindIII bands of pRjaPRC193 comigrate very closely, it was essential to use the plasmid as a molecular weight standard with gels containing cloned inserts. It is important to note that under the gel conditions used in this study, small bands less than a Kb in size are either diffused near the bottom of the gel or have migrated completely off the gel. In terms of physical mapping, this did not present a problem, but may be important for future, detailed, mapping of specific regions. In general, especially with the wide range of fragments encountered in this study, the 0.7% gels

Figure 2. Cosmid clones of pRjaPRC193 digested with HindIII A: lane 1, pMA115; lane 2, pMA112; lane 3, pMA106; lane 4, pMA116; lane 5, pMA117; lane 6, pMA118; lane 7 and 14, pRjaPRC193; lane 8, pMA125; lane 9, pMA126; lane 10, pMA135; lane 11, pMA134; lane 12, pMA133; lane 13, pMA142; lane 15, pMA129; lane 16, pMA139; lane 17 pMA119; lane 18, pMA137; lane 19, pMA140. B: Lane 1, pMA123; lane 2, pMA135; lane 3, pMA127; lane 4, pMA137; lane 5, pMA124; lane 6 and 13, pRjPRC193; lane 7, pMA101; lane 8, pMA105; lane 9, pMA119; lane 10, pMA167; lane 11, pMA169; lane 12, pMA171, lane 14, pMA113; lane 15, pMA130; lane 16, pMA131; lane 17, pMA146; lane 18, pMA120; and lane 19, pMA151.



Cosmid Clones of pRjaPRC193

worked well in separating fragments 49 to 1 Kb in size. The most accurate range of size determination in these gels was approximately 15 to 2 Kb.

Many of the cosmids in Figure 2 were grouped and placed in gels together on the basis of having overlapping fragments with other clones. The size of individual HindIII bands may be found in Table I, while the contents of HindIII inserts, as well as the size of each insert, are listed in Table 2 for each clone. 76 of the 111 cosmid DNA clones examined are listed in Table 2. A wide range of insert size exists for the clones: only 4.4 Kb in clone pMA156 and 28.7 in the largest clone, pMA121. The average insert size was 21.1 Kb with a standard deviation of 4.2. It appears from this information that fragments approximately 21.1 Kb were the most abundant partial-digest products available for ligation.

The reason 76 out of 111 cosmid clones are listed in Table 2 is as follows. First, many clones had identical inserts and only need to be represented by a single clone; second, some clones did not contain contiguous sequences, but rather had ligated fragments due to artifacts in the ligation and packaging procedure. Restriction digests of these clones with other enzymes confimed the DNA was not representative of adjacent DNA in pRjaPRC193. For example, cosmid pMA142 contains two fragments which hybridize to a nodulation probe, but are known by other evidence to be widely separated on the map of pRjaPRC193 (these data are discussed later in this section). Such

Table 2. Cosmid clones containing pRjaPRC193 DNA

Plasmid	HindIII fragments[a]	Insert size (Kb)
pMA101	5a,11a,32a,46	23.8
pMA102	12a,20a,21a,b,30,44	25.7
pMA103	11a,b,24a,28,37	25.2
pMA104	5a,11a,33,42	24.2
pMA105	16a,26,27,31a,32a,40	20.7
pMA106	11c,15a,21c,34,35	23.9
pMA107	5a,24c,42,44	18.7
pMA108	16a,19a,26,32a,38	19.8
pMA109	5a,33,34b,36a	19.2
pMA110	5a,11a,40,41,44,48	24.8
pMA111	16a,19a,24a,26,39,46	20.8
pMA112	6,21c,29	18.3
pMA113	4,20b,45	18.9
pMA114	11c,15a,21c	19.2
pMA115	6,10a,36a,41	22.9
pMA116	11c,15a,34b,35,38	21.5
pMA117	16b,25,34b,35,38,40	23.9
pMA118	16b,23,24b,25,35,38,40	20.1
pMA119	15b,20b,26,31b,46	19.4
pMA120	6,10a,36a	21.2
pMA121	5b,8,18a,44	28.7
pMA122	3,14,43	22.2
pMA123	11a,25,28,36b,37	19.2
pMA124	10b,13,21a,34a,46	23.8
pMA125	16b,24b,26,35,38,40	19.8
pMA126	16b,26,34b,38,40	16.1
pMA127	5a,11a	20.0
pMA128	11c,15a,21c,34b	21.6
pMA129	13,19b,22,31b,46,48	21.0
pMA130	8,21a,24c,37	20.1
pMA131	7,10b,23,24a,40	28.4
pMA132	5b,18a	17.6
pMA133	8,18a,25,39	20.9
pMA134	7,8,25,39	25.8
pMA135	11a,12b,24a,37,40,41	25.0
pMA136	7,23,24b,38,39	22.2
pMA137	26,27,32b,38,46	12.8
pMA138	10a,11a,36,40a,b,41	23.8
pMA139	4,20b,22,32a,45	25.8
pMA140	16a,19a,27,32b,38,	19.7
pMA141	16a,19a,24a,b,27,38	24.6
pMA142	5b,18a,19b	22.8

[a]HindIII fragments are ordered by size and values are given in Table 1; letters after numbers distinguish co-migrating fragments and are described in the text.

Table 2. (Continued)

Plasmid	HindIII fragments[a]	Insert size (Kb)
pMA143	8,25,39	15.3
pMA144	9,22,31b	15.9
pMA145	11c,15a,20b,21c,45	25.4
pMA146	15b,22,26,31a,b,32a,b,46	25.7
pMA147	5b,11a,40	21.8
pMA148	15a,22,31a,b,32a,46	19.5
pMA149	5b,18a,24c,44	22.7
pMA150	16b,23,26,27,32b,35,36a	24.5
pMA151	8,11x,24x,36x	23.8
pMA152	12b,24,a,b,37,40a,b,41	22.6
pMA153	3,14,32a,37	25.5
pMA154	3,14,43	22.2
pMA155	7,23,24a,b	22.1
pMA156	21x	4.4
pMA157	5b,18a,24c,42,44,48	24.3
pMA158	7,23,24a,40	20.1
pMA159	16a,19a,24a,26,27,33,41	26.6
pMA160	11c,15a,21c,34b	21.6
pMA161	7,8,24a,40a,41	27.6
pMA162	7,20x,23,24x	18.3
pMA163	16a,19a,34b,37	16.1
pMA164	1,26,31b,46,48	26.1
pMA165	11x,40x,46	11.0
pMA166	11a,37,40a,41	13.6
pMA167	12a,20a,21a,30	20.0
pMA168	26,27,37,38,40a,46	14.0
pMA169	10b,13,19b,34a	23.4
pMA170	11a,25,28,36b	17.1
pMA171	9,16b,22,31a	22.3
pMA172	9,15a,21c	20.0
pMA173	14,25,32x,39	15.2
pMA174	9,17,18b,22,31b	27.5
pMA175	12b,24a,b,37,40	19.2
pMA176	4,24x	16.5

clones may however be fortuitous events and a few such examples have been listed in Table 2. In general, clones with many similar HindIII fragments in common provided strong evidence for a given region. An example of this is the cosmids in lanes 3-6 and 8-9 of Figure 2A. These cosmids are: pMA106, pMA116, pMA117, pMA118, pMA125, and pMA126, respectively, in the same order as shown on the gel. The distance in Kb these clones cover is 45.5 Kb, or 13% of the plasmid. The position of these cosmids with respect to a physical map of a large region of pRjaPRC193 is shown in Figure 9.

3. Restriction enzyme analysis of cosmids

An examination of Table 1 shows that HindIII digests pRjaPRC193 DNA twice as frequently as the enzymes HpaI or KpnI. Actually, HindIII digests the plasmid into at least 60 fragments, but approximately 15 or so of these fragments are smaller than 1 Kb in size and their exact size could not be determined under the gel conditions used in this study. The important factor here is that the enzymes HpaI and KpnI recognize pRjaPRC193 digestion sites less often than HindIII and can be used effectively in mapping. Single digests with these enzymes were useful in determining if a given clone had a HpaI or KpnI restriction site or fragment. This information contributed to the determination of overlapping clones. Figure 3 contains photographs of gels with cosmid DNA digested with only one of the enzymes, and Table 3 a list of the HpaI and KpnI bands found in various inserts. Since HpaI has a single

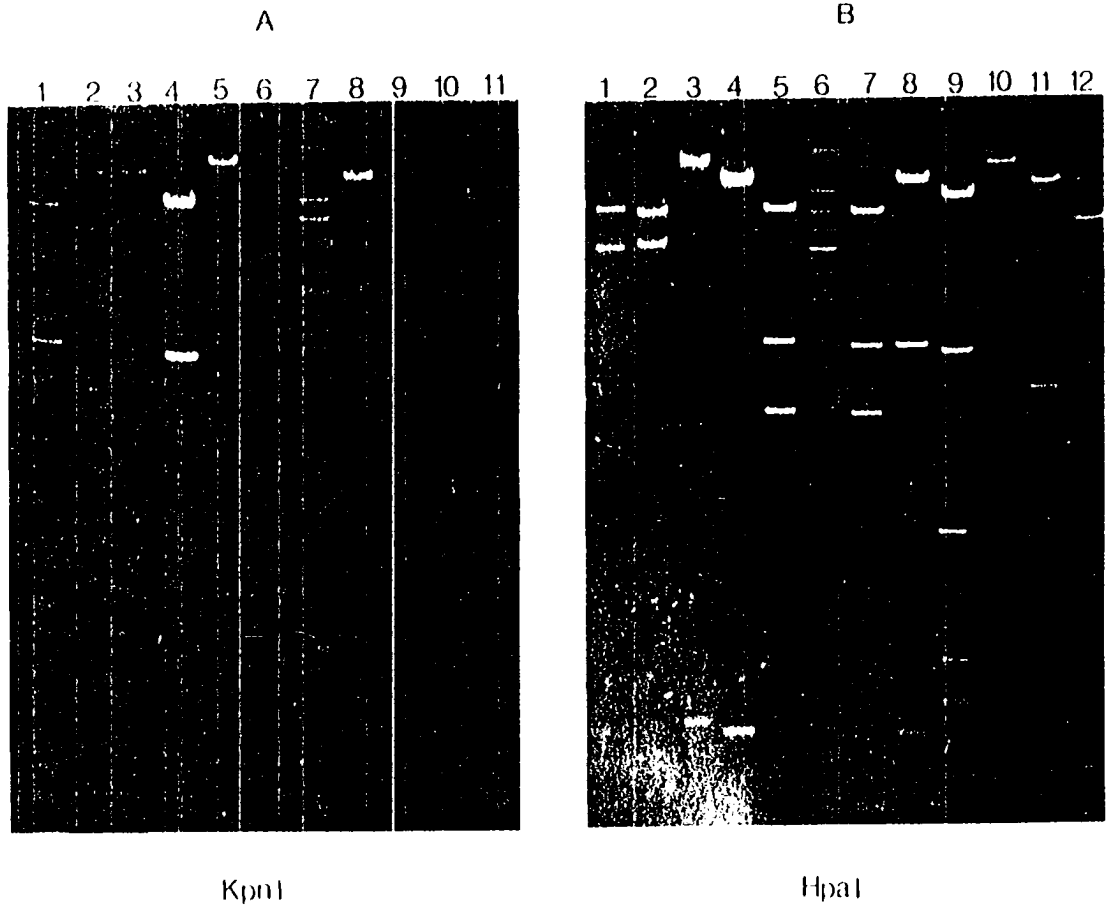
Table 3. HpaI and KpnI inserts in various pMA-clones[a].

HpaI		KpnI	
1.	n.d.[b]	1.	n.d.
2.	n.d.	2.	n.d.
3.	n.d.	3.	n.d.
4.	n.d.	4.	n.d.
5a,b	n.d.	5a,b,c	pMA101 (5a)
6.	pMA164	6.	pMA105, pMA134
7.	n.d.	7.	n.d.
8.	n.d.	8.	pMA164
9.	pMA103	9a,b	pMA115, pMA120
10.	pMA110, pMA135	10.	pMA108, pMA140
11a,b	pMA115 (11a)	11.	pMA121
12.	n.d.	12.	n.d.
13.	pMA140, pMA141	13.	pMA112, pMA174
14.	pMA103, pMA135	14.	n.d.
15.	pMA174	15.	pMA105, pMA145
16.	pMA119, pMA146	16.	pMA117, pMA118, pMA125, pMA126
17.	n.d.	17.	n.d.
18.	n.d.	18.	pMA144
19.	pMA119, pMA146, pMA158	19a,b	pMA101, pMA107, pMA111, pMA127 (19a); pMA140, pMA141, pMA163 (19b)
20.	pMA146		
21.	pMA135, pMA152	20.	pMA124, pMA129, pMA169
		21.	pMA113, pMA145

[a]Cosmid clones were digested with each enzyme.

[b]n.d. none detected.

Figure 3. Cosmid clones of pRjaPRC193 digested with KpnI (A) and HpaI (B) A: lane 1, pMA120; lane 2, pMA121; lane 3, pMA137; lane 4, pMA140; lane 5, pMA134; lane 6, pMA123; lane 7, pRjaPRC193; lane 8, pMA125; lane 9, pMA126; lane 10, pMA117; and lane 11, pMA129. B: lane 1, pMA134; lane 2, pMA118; lane 3, pMA161; lane 4, pMA137; lane 5, pMA103; lane 6, pRjaPRC193; lane 7, pMA135; lane 8, pMA110; lane 9, pMA146; lane 10, pMA171; lane 11, pMA127; and lane 12, pMA147.


A
1 2 3 4 5 6 7 8 9 10 11
B
1 2 3 4 5 6 7 8 9 10 11 12
Kpn I
Hpa I

site in the vector pVK102, and KpnI does not, each set of digestions will be considered separately.

The cosmid vector contains no recognition sequences for the enzyme KpnI. Cosmids digested with KpnI may have the following band pattern on gels. First, if no KpnI sequence is found in the insert, then the entire cosmid will remain undigested, and linear, open circular, and super coiled DNA is seen on subsequent gels. An example of this is the KpnI digest of cosmid pMA124 (Figure 3, lane 6). The recognition of a single KpnI site in the insert DNA results in a linear fragment. Several examples of this are the cosmids pMA132, pMA135, and pMA129 (Figure 3, lanes 3,5, and 11, respectively). The recognition of two or more KpnI sequences results in the 'liberation' of KpnI fragments from the insert DNA. Such fragments are very valuable as they can orient the digest patterns. That is, if a 4.3 Kb KpnI fragment is liberated from an insert, then one or more HindIII bands must contain KpnI site(s) exactly 4.3 Kb away from each other. This is exactly what has occurred in clones pMA125, pMA126, and pMA117 (Figure 3, lanes 8-10). In these clones, HindIII bands 16 and 25 contain KpnI sites, determined by double digests discussed in the next section, and these bands must be adjacent and accommodate a 4.3 Kb KpnI band (Figure 9). If three KpnI sites are located in an insert, then two KpnI bands are liberated as is the case for pMA140 (Figure 3, lane 4). In all the clones examined in this study, none contained more than 3 KpnI sites per cosmid.

Since HindIII inserts may be as large as 28 Kb, as seen in clone pMA121, as many as 24 KpnI bands may be liberated from inserts of the clone bank. In actuality, only 15 different KpnI bands were liberated from inserts. Twenty-nine of the 111 cosmids had KpnI bands liberated from inserts and the largest KpnI band was only 17.8 Kb. These facts are not too surprising however, in that many clones contained a single site in the inserts and therefore could not liberate a fragment. Since the average insert size was roughly 21 Kb, a KpnI site in the middle of this DNA section would only leave about 10 Kb of DNA on each side of the recognition sequence. An example of this is the large insert in pMA121 (Figure 3, lane 2). The insert DNA liberated KpnI band 11 but also contained 13.5 Kb of insert DNA on the left side of the clone (Figure 9), which did not contain KpnI recognition sequences. It turns out from hybridization data, discussed in a future section of this chapter, that KpnI band 3 occupies 13.5 Kb of pMA121 and extends and overlapps with other cosmids but, is not liberated from any particular insert.

In much the same way, HpaI was used to liberate fragments from cosmid clones. This enzyme digests pRjaPRC193 just as infrequently as KpnI and provides valuable information about overlapping sequences. A complication exists however, in that HpaI recognizes a sequence in the pVK102 vector 2.3 Kb from one of the HindIII vector-insert junction

sites. If an insert has no HpaI site, a linear band will result on an agarose gel due to a single cut in the pVK102 vector. Insert DNA with a single site liberates a band but this is due to recognition of a HpaI site in the vector and in the insert. Because these bands do not represent true HpaI fragments, the bands will be referred to as 'pseudobands' in this section. Pseudobands have an advantage however, in distinguishing the orientation of an insert with respect to the vector. This information was effectively used in conjunction with HindIII-HpaI double digests discussed in the next section. Only when two or more HpaI sites exist in the insert DNA, will a HpaI band will be 'liberated' from the cosmid clones.

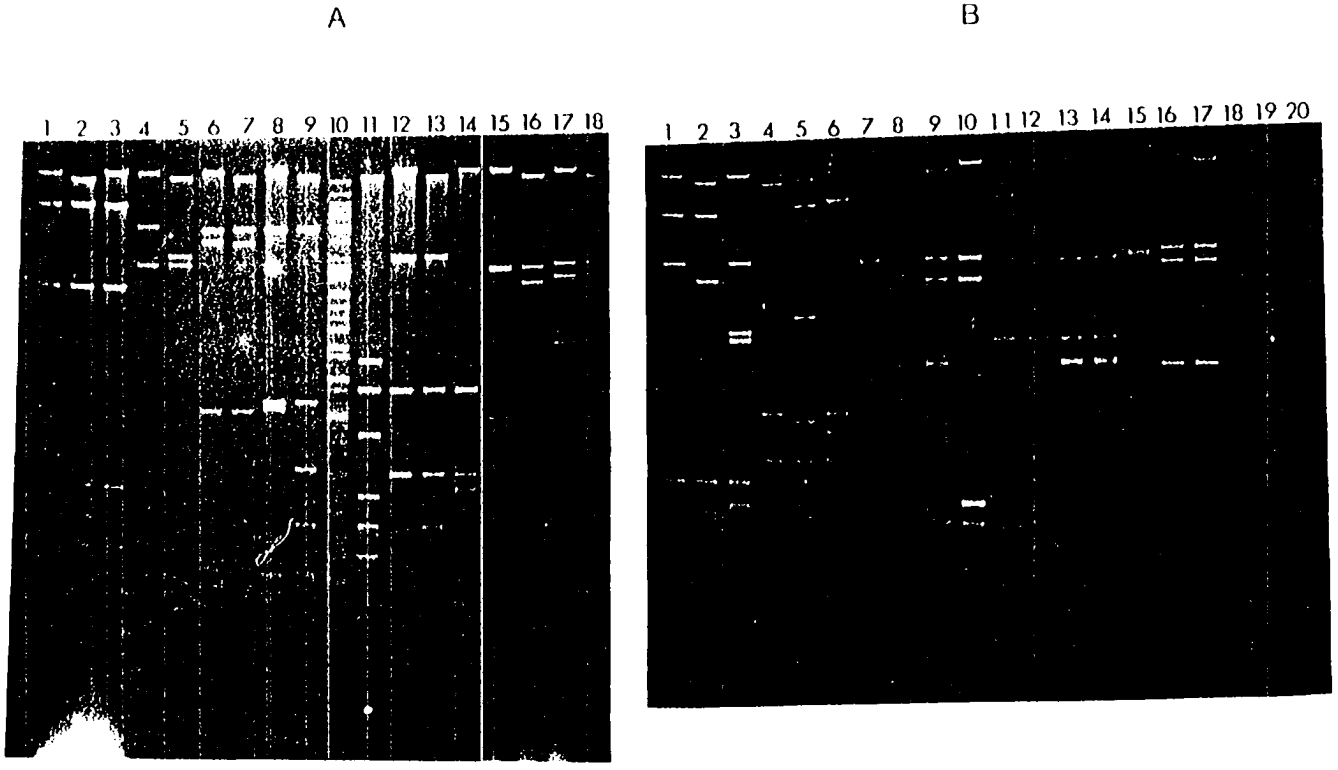
Figure 3B shows an agarose gel of Rhizobium cosmids digested with HpaI. The plasmid pRjaPRC193, also digested with HpaI, was used to determine HpaI liberated fragments. Cosmids were identified without HpaI sites in the insert DNA (Figure 3, lanes 10,12). Most cosmids appear to have a single HpaI recognition in the insert DNA and two bands are seen (Figure 3, lanes 1-4, and 11). Cosmids with two sites in the insert DNA provided bona fide HpaI bands and pseudobands (Figure 3, lanes 5, and 7-9). An example of information gained from these digestions with HpaI is the analysis of cosmid pMA146. The cosmid pMA146 produced five fragments when digested with HpaI (Figure 3, lane 9). The largest band (band 1) is composed of pVK102 vector and part of the HindIII insert. The next largest band is approximately 10.5 Kb and is a pseudoband. In this case, the pseudoband migrates

between HpaI bands 10 and 11 and clearly is not a HpaI liberated fragment. This pseudoband must have a HpaI site 2.3 Kb deep into the pVK102 vector; that is, 2.3 Kb to the vector side of a HindIII vector-insert site. The other 8.2 Kb of the 10.5 Kb pseudoband is insert DNA extending from the HindIII insertion site to the first insert HpaI site. The third, forth, and fifth largest bands are HpaI bands 16, 19, and 20, respectively. The order of these HpaI bands was determined by double HindIII-HpaI digestions. The orientation of this cosmid must therefore be: the largest vector band, followed by the pseudoband, which contains 2.3 Kb of vector DNA, and then HpaI bands 16, 19, and 20. Unidentified insert DNA follows the HpaI liberated bands and is part of the vector DNA which contains a HindIII site. The cosmid pMA146 has been assigned to a specific location on the physical map of pRjaPRC193 (Figure 9). Pseudobands in different cosmids are more difficult to analyze due to comigration with real HpaI fragments of pRjaPRC193 and double digestions with HindIII-HpaI were necessary to identify real HpaI bands (Figure 3, lanes 5 and 7).

4. Double digests of cosmid DNA

Figure 4 contains photographs of gels containing single and double digests of pMA-cosmids. Cosmid pMA101 is a good example of the information gained from double digest analysis (Figure 4B, lanes 1-3). Hybridizations with this DNA region are discussed in the next section. As shown in Table 2, pMA101 contains a 23.8 Kb insert with the

Figure 4. Cosmid clones of pRjaPRC193 digested with HindIII, HindIII-HpaI, and HindIII-KpnI double digestions A: lanes 1, 2, 3, contains pMA153 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively; lanes 4, 5, contains pMA115 digested with HindIII, and HindIII-HpaI, respectively; lanes 6, 7, contains pMA134 digested with HindIII, and HindIII-HpaI, respectively; lanes 8, 9, contains pMA136 digested with HindIII, and HindIII-HpaI, repectively; lane 10, pRjaPRC193 digested with HindIII; lane 11, pMA140 digested with HindIII-HpaI; lanes 12, 13, 14, contains pMA144 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively; lanes 15, 16, contains pMA103 digested with HindIII, and HindIII-HpaI, respectively; and lanes 17, 18, contains pMA169 digested with HindIII, and HindIII-HpaI, respectively. B: lanes 1, 2, 3, contains pMA101 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively; lanes 4, 5, 6, contains pMA164 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively; lanes 7 and 15, HindIII digested pRjaPRC193; lanes 8, 9, 10, contains pMA106 digested with HindIII, HindIII-HpaI, and HindIII-KpnI; lanes 11, 12, contains pMA113 digested with HindIII, and HindIII-HpaI, resectively; lanes 13, 14, contains pMA102 digested with HindIII, and HindIII-HpaI, respectively; lanes 16, 17, contains pMA124 digested with HindIII, and HindIII-HpaI, respectively; lanes 18, 19, 20, contains pMA145 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively.



Double Digests: HindIII-HpaI, HindIII-KpnI

following HindIII fragments: 5a, 11a, 32a, and 46. Digestion of pMA101 with HindIII and subsequent gel electrophoresis provides separation of individual bands (Figure 4B, lane 1). A double digestion of pMA101 with HindIII and HpaI directly shows which HindIII bands are digested with HpaI (Figure 4B, lane 2). The largest band is a truncated part of the pVK102 vector; a 2.3 Kb HindIII-HpaI vector fragment, described in the previous section, is also produced by this double digest. The next largest band migrating in the gel is HindIII band 5a. This fragment does not contain a HpaI site and therefore remains the same size. The third largest band is HindIII band 11a which contains a single HpaI site. A 7.0 Kb and 1.0 Kb fragment are produced as can be seen in the gel (Figure 4B, lane 2). This characterizes other HindIII band 11s with the same HpaI digestion pattern as 11a and assists in determining overlapping DNA sequences. This does not however, guarantee that two band 11as are the same fragment because 11a could be duplicated elsewhere in the plasmid pRjaPRC193, and show the same digestion pattern. Continuing with the HindIII-HpaI digest of pMA101, it is clear HindIII band 32a does not digest with HpaI (Figure 4B; lane 2). Thus, the cosmid pMA101 contains a single HpaI site located in HindIII band 11a.

At this point, it is important to note that a given HindIII band may contain a HpaI site very near its end. If this is the case, the fragment would appear to remain the same size and a tiny end piece would migrate off the gel. Such a situation could only be resolved by

single digests of a cosmid with HpaI as described in the previous section. Therefore, a combination of various single and double digestions are very valuable for accurate restriction mapping analysis of large inserts. An examination of pMA101 with HpaI only indicated a single site occurred in the insert DNA, which verifies the above observations.

Double digests with HindIII-KpnI of pMA101 shows the effect of having a KpnI fragment present in the 23.8 Kb HindIII insert. Three fragments corresponding to the digestion of the HindIII band 5a fragment by KpnI are seen in the double digestion, and have molecular weights of 5.1, 4.9, and 2.3 Kb (Figure 4B, lane 3). The other HindIII bands of the insert do not digest with KpnI. A rapid scan of the molecular weights in Table 1 shows that the only band corresponding to the molecular weight of a KpnI band is the KpnI band 19a, a 2.3 Kb fragment. The 5.1 and 4.9 Kb bands must be on either side of the 2.3 Kb band for its liberation by KpnI. A single digest of pMA101 showed two bands were liberated from the cosmid, namely, KpnI band 5a and 19a (Table 3). A very small HindIII band containing a KpnI site is at the bottom of the lane containing HindIII digested pMA101, and is missing in the lane with the double digest HindIII-KpnI (Figure 4B, lanes 1 and 3, respectively). Since HindIII band 5a and this very small band digest with KpnI, the large KpnI band 5a must span 17.8 Kb of the pMA101 insert. Therefore, the KpnI band 5a assists in the orientation of HindIII fragments in pMA101. In this case, not only was the band

location of KpnI sites determined, but a KpnI fragment was released from HindIII band 5a. This clearly identifies one of the 12 Kb HindIII bands as 5a. Together with the HindIII-HpaI double digest information, the evidence provided was used in determining the map order of pMA101 (Figure 9).

Evidence of linkage relationships between cosmids with overlapping sequences has been determined to a great extent by the single and double digests described above. This has been especially important in situations where the overlapping HindIII fragments have similar molecular weight counterparts, that is, multiple bands. For example, clones pMA106 and pMA145 both have HindIII bands 11x, 15x, and 21x in common (x means the identity of the band is unknown for multiple bands). An examination of HindIII-HpaI digests of the two cosmids does not differentiate between the multiple bands present in each insert (Figure 4B, lanes 9 and 19). A HindIII-KpnI digest determines HindIII band 21x, which can now be termed 21c, has the same digest pattern in the two cosmids (Figure 4B, lanes 10 and 20). The 4.4 Kb HindIII band 21c is digested by KpnI into a 2.4 and 2.0 Kb fragment. Here, it is important to observe the increased intensity of the double digest of the 2.4 Kb HindIII-KpnI fragment which comigrated with the HindIII band 34b (Figure 4B, lane 10). A comparison of the HindIII-KpnI digest of HindIII band 21c helps to confirm this observation (Figure 4B, lane 20). A similar observation was found with the HindIII-KpnI double digest of cosmid pMA144 (Figure 4A, lanes

12-14).

The cosmid pMA153 does not appear to digest with HindIII-HpaI or HindIII-KpnI (Figure 4A, lanes 1-3). This cosmid is of particular importance because it contains two HindIII bands, 3 and 14, which hybridize with a nifW probe (discussed in the next section). When digested only with KpnI, pMA153 does produce a single band on a gel. This suggests a KpnI site exists in the insert DNA. A close comparison of HindIII band 3, double digested with HindIII-KpnI, shows a slight reduction of HindIII band 3 (Figure 4A, lane 3). Another example of this situation was encountered with cosmid pMA134, although the reduction in size of HindIII band 8 was clearer (Figure 4A, lanes 6 and 7). A related problem occurred in cases where a double digest did not appear to cut a given fragment, but a new, smaller sized band appeared. This was observed with pMA103 when double digested with HindIII-HpaI (Figure 4A, lanes 15 and 16). It was originally thought only one HindIII band 11 existed in the cosmid insert. The double digest revealed the presence of one HindIII band 11, without a HpaI site, and hence termed 11a, and a band 11 which digested with HpaI, HindIII band 11b. Most of the time two comigrating bands are easily detectible, but in this case the observation went unnoticed until the HindIII-HpaI double digestion.

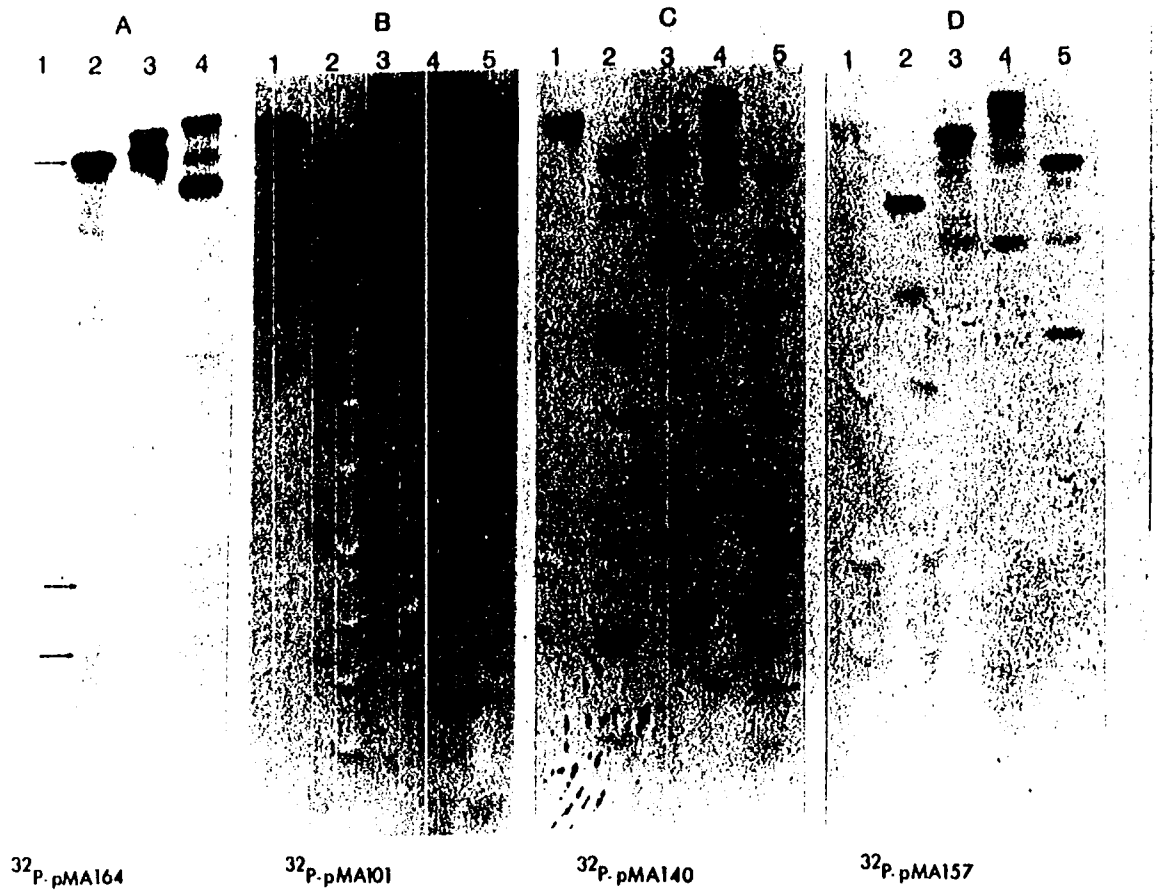
5. Southern hybridizations for mapping

Another tool used in physically mapping DNA is Southern

hybridizations (Southern, 1975). ^{32}P-labeled cosmids were hybridized to Southern transfers containing: HindIII digested Lambda DNA; HindIII digested pRjaPRC193; HpaI digested pRjaPRC193; KpnI digested pRjaPRC193; and HpaI KpnI double-digested pRjaPRC193 (Southern blots in Figures 5A and 6C did not contain the double-digest). These blots will be referred to as 'pRjaPRC193 Southern blots' unless otherwise noted. An agarose gel containing these digests has been shown previously in this section (Figure 1, lanes 1-5). Replicas of these digests were used in the Southern hybridizations. In principle, a sequence of labeled, ssDNA should bind to homologous ssDNA bound to filters, as previously described in the first two chapters. In mapping analysis, the hybridizing DNA sequences can be used to determine overlapping sequences amoung clones, identify similar sequences digested with other enzymes, and assign known gene sequences, such as nif genes, to a particular location. This is not the case for the plasmid pRjaPRC193.

The hybridizations described below show that many repeated sequences exist in pRjaPRC193. A given hybridization using one of the pMA-cosmids showed homology not only to its own insert, but also to many other restriction fragments. While the HindIII bands in the insert are known, the strategy here was to use hybridizations for identifying HpaI and KpnI bands which overlap the insert. Hybridizations using the structural nif genes and nodulation genes as probes showed these sequences are repeated once each in the plasmid

Figure 5. Southern hybridizations using different cosmid clones as probes to digested pRjaPRC193 Exact transfer membrane replica's of lanes 1-4 and 1-5 of Figure 1 were used in the hybridizations. A: lane 1, Lambda DNA digested with HindIII, and lanes 2, 3, and 4, contains pRjaPRC193 digested with HindIII, HindIII-HpaI, and HindIII-KpnI, respectively. The hybridization probe is pMA164. B: lane content the same as A, except that HpaI-KpnI digest of pRjaPRC193 is in lane 5. The hybridization probe is pMA101. C: lane content the same as B. Hybridization probe is pMA140. D: lane content is the same as B and C. Hybridization probe is pMA157.


A
B
C
D
1 2 3 4
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
32P-pMA164
32P-pMA101
32P-pMA140
32P-pMA157

pRjaPRC193 and confirms the work of Prakash and Atherly in this laboratory (Department of Genetics, Iowa State University, personal communication). Although repeated sequences are more difficult to map, these symbiotic gene sequences were assigned to specific locations on the physical map. Other sequences are also repeated throughout the plasmid, although their genetic function is unknown. The aspect of repeated DNA sequences is more thoroughly examined using a novel hybridization method in the following section on Southern Cross restriction mapping. Nonetheless, some of the information gained from the Southern hybridizations was useful and is described below.

The first Southern hybridization example is with cosmid pMA164. The reason this clone was examined using these procedures is that no other cosmid was found with HindIII band 1 for use in overlap analysis. The autoradiograph of a 'pRjaPRC193 Southern blot' with ^{32}P-labeled pMA164 is shown in Figure 5A. The HindIII bands 1, 26, 31, and 46, from the insert in pMA164 produce bright hybridization signals with HindIII digested pRjaPRC193 (see arrows in Figure 5A). The other pRjaPRC193 HindIII bands which hybridized are sequences in pMA164 that are homologous to other regions of the plasmid. The HpaI digested pRjaPRC193 bands 1, 3, 5, 6, 13, 15, 16, 18, 19, all hybridize with the probe, although to varying degrees (Figure 5A). The KpnI digested pRjaPRC193 bands also hybridized strongly with the probe, and they include KpnI bands 2, 7, 8, 13, 14, and 17. It was known from evidence presented earlier that HpaI liberates HpaI band 6, and KpnI liberates

KpnI band 8 from pMA164. These bands hybridize and show strong signals with pRjaPRC193 (Figure 5A). The liberated bands had already determined the orientation of the HindIII bands in the insert of pMA164 (Figure 9). The important questions however, are which HpaI and KpnI bands overlap the HindIII insert in pMA164. To answer this, the HpaI and KpnI bands which produced the greatest amount of intensity, like the HindIII hybridizing fragments, were screened for their potential as overlapping fragments with pMA164. HpaI band 3 hybridized strongly and fit, with respect to HpaI and KpnI digests, to a clone previously described (Figures 5A and 9). KpnI band 14 also strongly hybridized and fit with clone pMA101 (Figures 5A and 9). In this way, bands which hybridized strongly were matched with possible adjacent clones.

Clone pMA101 was labeled and hybridized to a 'pRjaPRC193 Southern blot' in the same way as the cosmid pMA164. This was done to confirm the observations made above for HpaI band 3 and KpnI band 14. These two bands did hybridize strongly and gave further support to the notion that pMA146 and pMA164 are adjacent to each other in pRjaPRC193 (Figure 5B). Other bands, not present in the insert, also hybridized to HindIII digested pRjaPRC193, and presumably to HpaI and KpnI digested pRjaPRC193 as well. The HpaI bands include: 3, 5, 7, 9 or 10, and 11. KpnI bands which hybridized with the cosmid are: 1, 2, 5, 14, and 19. This evidence supports the information obtained from the

liberation of KpnI band 5a and 19a from pMA101 and shows KpnI band 14 probably overlaps the cosmids pMA101 and pMA164.

A problem is encountered however, in using bands which only hybridize strongly as the basis for determining true, overlapping sequences. An insert such as that found in pMA164 may only have a small region of homology with an overlapping HpaI or KpnI fragment. This occurs on the side of pMA164 containing a HpaI site for HindIII band 26. Only a small amount of DNA remains distal to the rest of the insert. Cosmid pMA146, described in the preceding section, liberates HpaI band 16 when digested with HpaI. Since both pMA164 and pMA146 have overlapping sequences, and pMA164 hybridized with HpaI band 16, the two cosmids are probably adjacent in pRjaPRC193 (Figures 5A and 9).

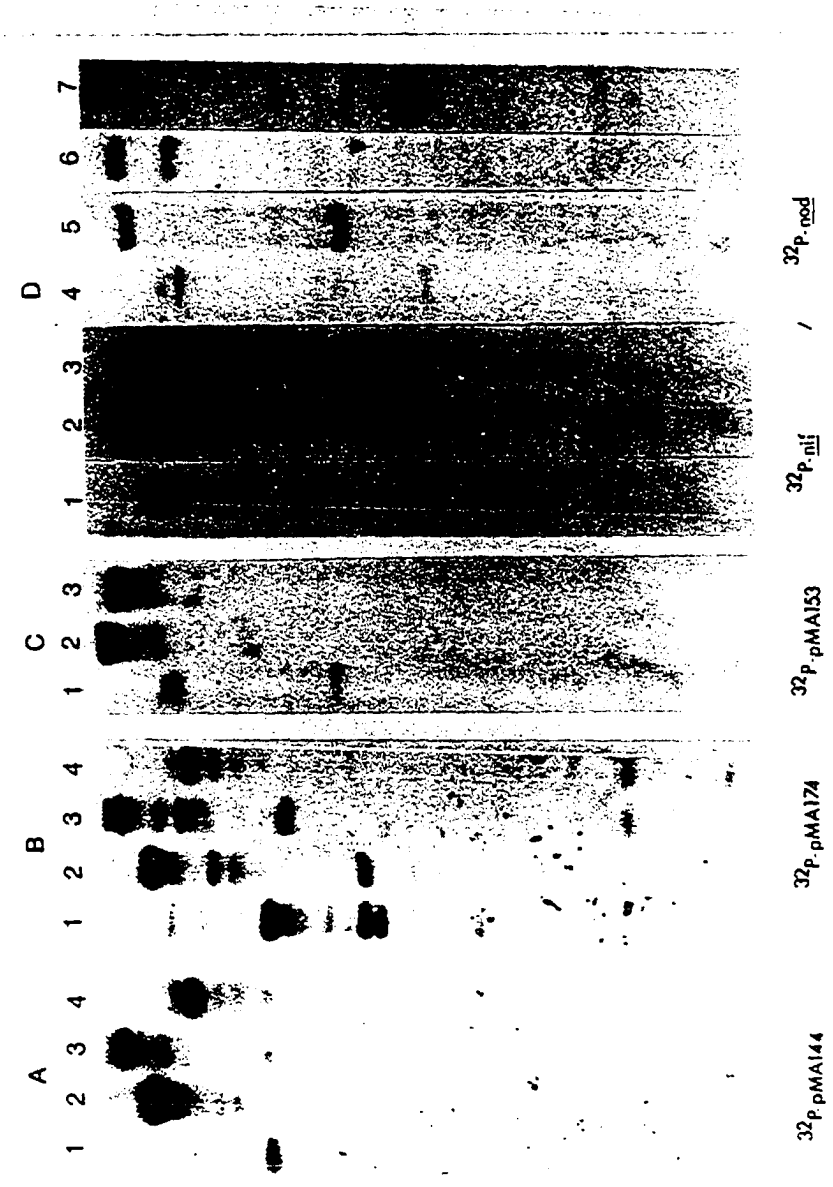
This type of analysis with other cosmids yielded the same kind of results. Hybridizations with cosmids pMA140 and pMA157 to 'pRjaPRC193 Southern blots' again showed homology with more sequences than those present in the inserts (Figures 5C and 5D). The data using pMA157 provided evidence for overlapping sequences near structural nif genes. Until the Southern blot was done using pMA157 as a probe, the KpnI overlap of pMA157 and pMA122 was unknown. In this particular case, the hybridization data clearly identified KpnI band 3 as the overlapping fragment (Figure 9). Hybridization using pMA140 as a probe only verified the HpaI and KpnI liberated fragments (Figures 5C and 9). Some of the other bands which hybridized were already assigned to

other clones; HpaI bands 16 and 19 hybridized but are located in the insert of pMA146. Other bands which hybridized with this clone could not be assigned to overlap with different clones. HindIII band 32b is a common band between clones pMA140 and pMA157 and could be partly responsible for the hybridization results obtained.

Hybridizations using the cosmid pMA144 supported evidence for overlapping sequences with pMA146 (Figure 6A). These cosmids had HindIII bands 22 and 31b in common. The 'left' side of pMA144 was unknown however, and a potential adjacent cosmid was hybridized to a stripped blot of the pMA144 hybridization (Figure 6B). This cosmid was pMA174 which contained three bands in common to pMA144, HindIII bands 9, 22, and 31b, and had in addition HindIII bands 17 and 18b. The hybridization result was generally agreeable, except that clone pMA174 showed homology with KpnI band 13 and pMA144 did not. Since the insert of pMA144 is presumedly included in pMA174, the result is not readily explainable.

The location of symbiotic genes was determined by hybridizations with cloned sequences. The properties of the nif and nod probes have been described in the previous two chapters. The structural nif genes were hybridized to a Southern blot containing HindIII digested pRjaPRC193 DNA in order to determine the fragments harboring nif gene sequences. Strong hybridization was observed with HindIII bands 2 and 3, while a moderate to low amount was seen with bands 14, 30, and 40 (Figure 6D). Clones pMA153 and pMA154 contain HindIII bands 3 and 14

Figure 6. Southern hybridizations using cosmid clones as molecular probes and examination of symbiotic gene sequences in various cosmids A: lane 1, Lambda DNA digested with Hind III; lanes 2, 3, and 4, contains pRjaPRC193 digested with HindIII, HindIII-HpaI, HindIII-KpnI, and HpaI-KpnI, respectively. The hybridization probe is pMA144. B: lane content is the same as A. Hybridization probe is pMA174. C: lane content is the same as the first three lanes in A. Hybridization probe is pMA153. D: hybridization of nif gene sequences to lanes 1, 2, and 3, containing pRjaPRC193, pMA153, and pMA154, respectively. Also, hybridization of nod gene sequences to lanes 4, 5, 6, and 7, containing pRjaPRC193, pMA106, pMA121, and pMA169, respectively.


A
B
C
D
1 2 3 4
1 2 3 4
1 2 3
1 2 3 4 5 6 7
32P-pMA144
32P-pMA174
32P-pMA153
32P-nif
32P-nod

(Figure 6D). Cosmids pMA102 and pMA167 had HindIII band 30; pMA117, pMA118, and pMA161, harbored HindIII band 40 (results not presented).

The 3.5 Kb nod probe from R. meliloti hybridized to HindIII bands 5b, 15a, and 19b (Figure 6D). Clone pMA157 contained the largest HindIII band, 5b (Figure 6D). Clones pMA172 and pMA169 carry HindIII bands 15a and 19b, respectively (Figure 6D). Therefore, the genes involved in symbiosis were first hybridized to HindIII digested pRjaPRC193 and then to selected clones which contained similar sized bands.

Hybridizations were performed with one of the cosmids carrying nif genes described above. The cosmid pMA153 was hybridized with a 'pRjaPRC193 Southern blot' and HpaI bands 1, 2, 7, 12, and 20, all showed homology. KpnI bands 2, 3, 5, and 15, also hybridized with the probe. Since a KpnI site had been found near the end of HindIII band 3, as previously described, some idea of the order in the insert was established. This clone was eventually assigned a position adjacent to clone pMA157, and was described in an earlier section (Figure 9).

6. Southern Cross restriction mapping

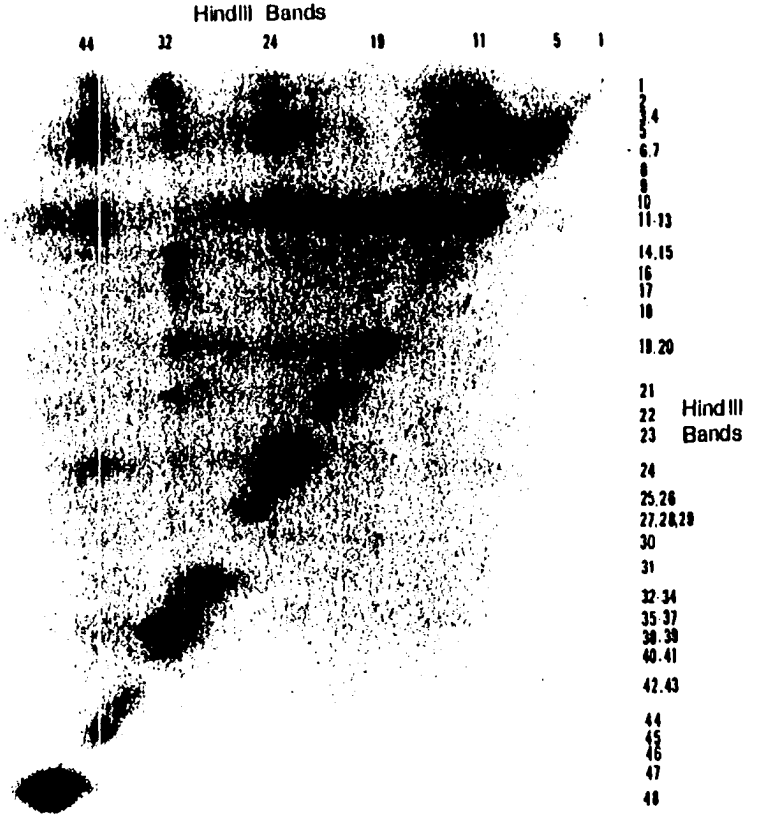
A new, novel procedure was used in mapping and determining repeated sequences in the plasmid pRjaPRC193. Basically, two previous methods, Southern hybridization and blotting, are employed at the same time such that a physical map of given piece of DNA, with two or more restriction enzymes, can be made. A packaged kit was purchased (New

England Nuclear) which included chemicals and instructions for this type of mapping (described in the Materials and Methods section). The enzymes HindIII and HpaI were used to map and verify overlapping sequences of pRjaPRC193 in this application of the Southern Cross method and in the determination of repeated sequences in the plasmid.

The procedure takes place in two stages: preparation of Southern blots, each containing a different digest of pRjaPRC193, and hybridization of homologous DNA on membrane filters in a sandwich type structure. pRjaPRC193 was digested with HindIII, and also with HpaI separately, and the resulting fragments were separated by gel electrophoresis, transferred to GeneScreen Plus hybridization transfer membrane (New England Nuclear) and allowed to bind. While these 'cold' transfers were taking place, a 'hot' transfer with ^{32}P end-labeled pRjaPRC193 was constructed with regular GeneScreen membrane. The key factor in the whole procedure is that the 'hot' transfer is not allowed to bind to the membrane. The labeled HindIII transfer was placed in hybridization conditions such that the fragments were free to 'diffuse' through the cold membranes, which were placed at a 90^{o} angle with respect to the labeled transfer membrane. Non-homologous DNA was removed and the resulting autoradiographs are shown in Figures 7 and 8.

The Southern cross restriction mapping method may best be illustrated by using your hands in the following analogy. Let your right hand represent the ^{32}P-labeled HindIII digest transfer. The

Figure 7. Southern cross hybridization with ^{32}P-HindIII digested pRjaPRC193 versus nonlabeled HindIII digested pRjaPRC193 Diagonal line represents single copy sequences, while hybridization signals apart from the diagonal line are repeated sequences.



Southern Cross Hybridization

^{32}P-HindIII vs HindIII pR$_j$aPRC193

index finger is the largest band, while the little finger represents the smallest band. With this hand palm up, place the left hand palm down, and at a 90^{0} angle over the right hand allowing contact between the fingers of each hand. The plasmid DNA on the left hand may represent a control, that is, a cold HindIII digest, or may represent a different enzyme for mapping purposes, in this case HpaI. Under hybridization conditions, the 'hot' HindIII bands from the right hand will diffuse up to and through the left hand, which has its DNA firmly bound. Only those places where homologous DNA intersects will dsDNA hybrids form. After the hybridization period, the hands are washed. The right hand will contain some labeled sequences which did not transfer, while the left hand can have two possibilities. First, if the DNA on the left hand is a HindIII control, then a diagonal line should be seen on an autoradiograph. That is, a spot will form where each similar, or homologous, finger comes into contact. The two index fingers will form a spot where they touch, and so on. A diagonal series of dots, due to hybridization signals at homologous intersects, will be seen in an autoradiograph of this control. Second, if the cold transfer on the left hand contains a different digest, such as HpaI digested pRjaPRC193, then a specific array of dots is expected corresponding to homologous sequences. Since the orientation of the largest DNA bands are known, the index fingers representing these bands, the relationship between two different digests can be established.

A very interesting result was obtained from the HindIII control (Figure 7). At least 15 hybridization spots were detected off the diagonal line. These signals represent homologous sequences that are repeated and distributed throughout the plasmid. It has been shown that both nif and nod sequences are repeated once each. Since the nif gene sequences are present mainly on HindIII bands 2 and 3, the bands are too close to show divergence from the diagonal line. However, hybridization spots can be seen between HindIII bands 5, 15, and 19, which contain sequences homologous to the nod probe. The genetic content of the other signals is unknown. A comparison of the results obtained for repetitive sequences using Southern blots, described earlier in this section, and these results suggest a small sequence may be repeated. It is possible it is an insertion sequence, but detailed analysis will be required to solve this dilemma.

The hybridization of HindIII labeled plasmid to HpaI digested pRjaPRC193 resulted in the autoradiograph seen in Figure 8. The hybridization signals have been examined and are listed in Table 4. An initial examination of the hybridization shows that many bands have considerable homology with each other. Here again, the problem of repeated sequences interferes with the mapping process. Even small HpaI bands show homology with many bands of HindIII digested pRjaPRC193. For instance, HpaI band 20, only 2.6 Kb in size, hybridizes with HindIII bands 11, 14 or 15, 24, 32, 39, and 40. Evidence presented earlier showed HpaI band 20 was contained in clone

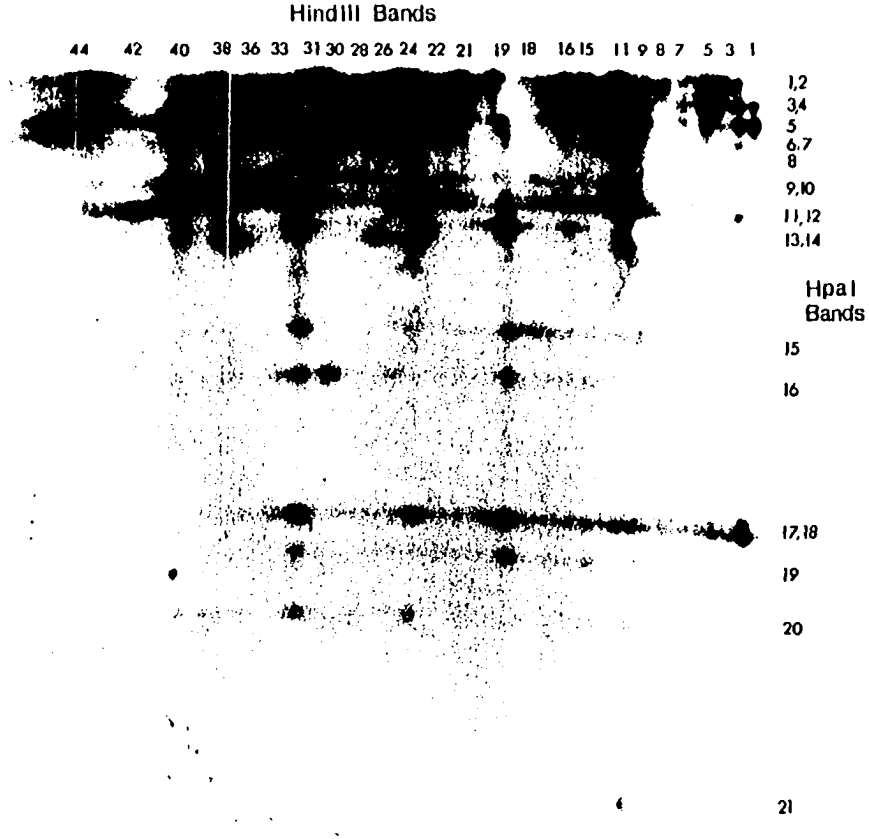
Table 4. Southern Cross restriction mapping of pRjaPRC193

HpaI[a]:	HindIII bands homologous to HpaI bands[b]:
1a,b	2,3,4,8,9,11 or 12,15,16,17,20,21,24,31,32,34,36,37,41
2.	5,8,9,15,16,21,23,24,31,34,35,38,43
3,4.	1,2.3,5,10,11,12,15,16,24,42
5a,b	1,2,5,9,10,11,12,15,16,19,21,22,23,24,25,30,31,32,35, 36,43,44
6.	1,26,31
7,8.	2,11,14,19,22,24,27,35,43
9.	11,18,20,32,33,40
10.	11,22,28,32,40
11a,b	6 or 7,11,19,24,32,38,40
12.	2,11,19,24,32,41
13.	16,19,32
14.	11,24,27,36
15.	2,5,19,24,32
16.	2,19,31,32
17.	2,19,24,32
18.	2,10,12
19.	2,14 or 15,19,32
20.	11,14 or 15,24,31,39,40
21.	11 or 12

[a]HpaI bands which migrated very close to each other, and could not be resolved with respect to hybridizations, are shown in pairs.

[b]HindIII digested pRjaPRC193 was end-labeled with ^{32}P.

Figure 8. Southern cross hybridization of ^{32}P-HindIII pRjaPRC193 versus nonlabeled HpaI digested pRjaPRC193. Hybridization signals show homologous sequences



Southern Cross Hybridization

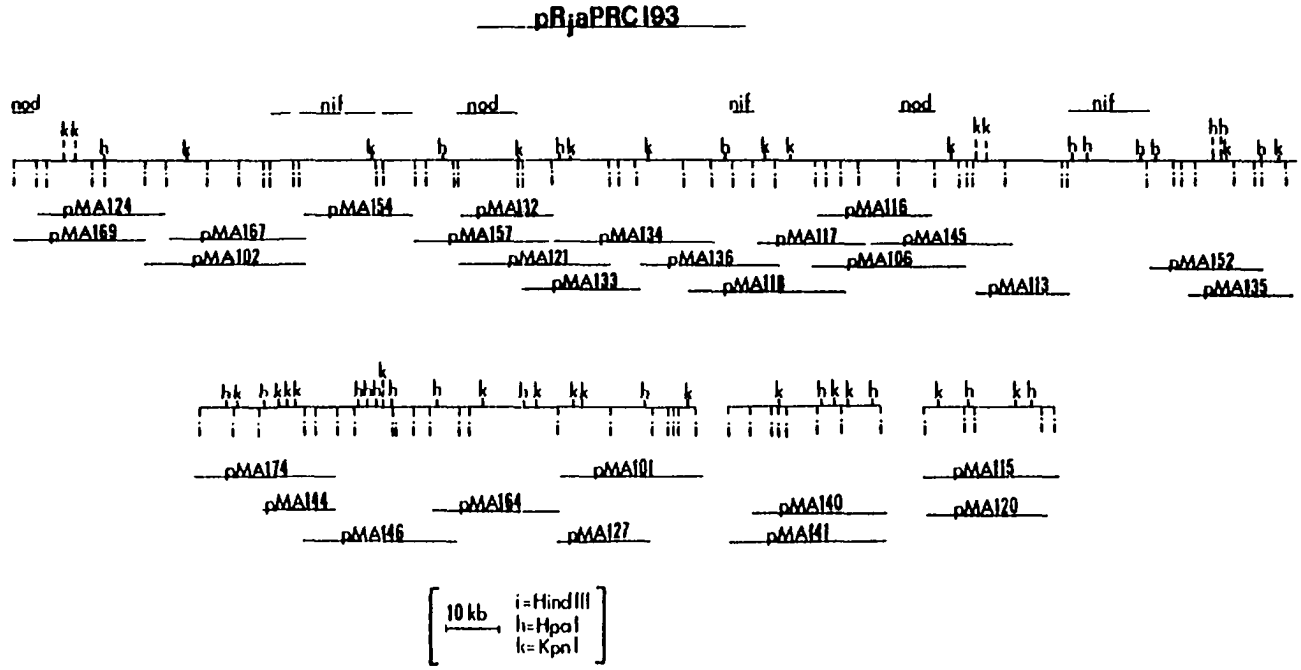
^{32}P-HindIII vs HpaI

pMA146. The true, homologous HindIII bands are therefore 15 and 32, as these are the only bands also present in the insert of pMA146.

Taking the repetitive sequences into account, it was possible to find a number of homologous, overlapping sequences with this procedure. The Southern cross data were consistent with information collected from restriction digestions of the cosmids. It was useful to check with these data when considering a given overlap of HindIII and HpaI fragments. Also, HindIII bands which did not hybridize with certain HpaI bands gave evidence that these DNA fragments could not overlap. This negative evidence was useful with double digest analysis of some cosmids where overlapping HpaI bands were being considered. Perhaps the best example of using this blot for mapping purposes is seen with HpaI band 13. Bright hybridization signals occur between this band and HindIII bands 16, 19 and 32. An examination of cosmids carrying these HindIII fragments shows that pMA140 and pMA141, described earlier, do in fact liberate HpaI band 13. HpaI band 13 overlaps HindIII bands 16 and 19 (Figure 9).

It appears from this use of the method that several improvements can be suggested. The HindIII digest produces fragments less than 18.5 Kb, which are suitable for transfer, but also contains at least 12 different comigrating fragments. In the example illustrated above with HpaI band 13, it is not readily known which of the double, HindIII bands 16 or 19 are homologous. However, with a DNA molecule 350 Kb in size, no enzyme was found which produced ideal-sized bands for

Figure 9. Physical and genetic map of large regions of pRjaPRC193 The restriction enzymes HindIII, HpaI, and KpnI were used to construct the physical map. Cosmid clones used for mapping were assigned to specific map locations. Nif and nod gene sequences and their locations are shown above the physical map.


pRjaPRC193
nod
nif
nod
nif
nod
nif
pMA124
pMA154
pMA132
pMA116
pMA169
pMA167
pMA157
pMA134
pMA117
pMA145
pMA102
pMA121
pMA136
pMA106
pMA152
pMA133
pMA118
pMA113
pMA135
pMA174
pMA101
pMA115
pMA144
pMA164
pMA140
pMA120
pMA146
pMA127
pMA141
10 kb
i=HindIII
h=HpaI
k=KpnI

transfer and yet had few doublets. An unsuccessful attempt was made to transfer ^{32}P end labeled HpaI digested pRjaPRC193 in another, similar Southern cross assay. Most of the large fragments did not appear to transfer through the GeneScreen Plus membranes, which suggests the limit of the size of the band transferred is probably close to 20 Kb. HpaI bands larger than HpaI band 6, which include bands 20 Kb and larger, account for well over 200 Kb of DNA, or nearly 62% of the plasmid. Also, it is imperative with this method that the hot digest contain many fragments for transfer to cold blots. The enzyme XbaI digests pRjaPRC193 into only eight fragments. If this was the hot transfer, very limited information would be obtained due to the few number of bands and the repeated sequences in pRjaPRC193. In addition, much information was obtained from the HindIII control assay due to the 44 or more bands present in the digest (Figure 7). Therefore, while there appears to be alternative suggestions, the enzyme HindIII remains an acceptable choice for this method. Since so much was known about the HindIII and HpaI digest patterns before the use of the Southern cross, it was valuable to use this information as an aid in the mapping process.

V. DISCUSSION

The goal of this study was three-fold: 1) to find the location of symbiotic genes in R. japonicum; 2) do DNA:DNA relatedness studies of the symbiotic genes and the DNA surrounding these regions; and 3) to produce a physical and genetic map of a R. japonicum symbiotic-coding plasmid. The first two aspects of the goal were accomplished and over 90% of the plasmid pRjaPRC193 was mapped.

Of the limited number of strains examined, it was interesting that structural nif and nod genes were found exclusively on the chromosome, or large megaplasmid, of slow-growing R. japonicum. In the fast-growing R. japonicum strains examined, these genes were found mostly on large plasmids, and in only one case on the chromosome or megaplasmid (Masterson et al. 1982). This suggests the symbiotic genes are recent introductions to the fast-growing strains and that slow-growing strains have been endowed with this property for a longer time. Radiation of plasmid-borne symbiotic genes, perhaps aided by conjugative plasmids, has been suggested by Ruvkun and Ausubel (1980) as an explanation for the diverse types of strains having the nitrogen-fixing, symbiosis properties.

The hybridization of nitrogen fixation and nodulation gene sequences to the DNA of slow- and fast-growing strains revealed their

location. Strong conservation of these genes occurs in R. japonicum with respect to their EcoRI restriction patterns. All the fast-growing strains have exactly the same hybridization pattern, even strain PRC193 which does not harbor nif or nod genes on its plasmids. Nodulation gene sequences in the fast-growers is conserved but show some strain-to-strain variation in their EcoRI restriction pattern. It appears the nodulation sequences are not as highly conserved as the nif sequences and perhaps reflects the differences in the roles of the protein products of each system in symbiosis. The slow-growing R. japonicum strains show a high degree of nif gene sequence conservation with respect to hybridizations of EcoRI-digested total DNA. Strains from many diverse locations showed the same nif hybridization profile. The hybridization of nod structural genes, namely the 3.5 Kb nod fragment from R. meliloti (Long et al. 1982), was radically different from the results obtained with the fast-growing R. japonicum strains. Hybridization was seen only when the nod probe was hybridized under reduced hybridizations. The EcoRI bands that hybridized showed a good amount of conservation between the slow-growing strains. One important concern is these particular hybridizations is the known presence of insertion sequences near symbiotic genes in R. meliloti (Ruvkun et al. 1982). If insertion sequences reside in or near symbiotic genes in R. japonicum, then hybridizations with nodulation probes must take this factor into account. Whether or not these hybridizations truly represent nodulation gene sequences is now being investigated in the

laboratory.

The identification and location of symbiotic genes in Rhizobium has involved transfer of plasmids, harboring the genes for symbiosis, and hybridization analysis with known genes as molecular probes. While these studies have contributed to the developing story of the plant-bacteria symbiosis, the sequences surrounding these genes have been virtually ignored. Much of the reason for this has been a lack of genetic markers and mutagenesis methods. Mutagenesis with the wide host range transposon Tn5 has greatly improved genetic studies in many different Rhizobium strains (Meade et al. 1982). A major aspect of this study has been an examination of the sequences surrounding the symbiotic genes by using various DNA sequences as molecular probes. To this end, the plasmid pRjaPRC193, from fast-growing R. japonicum strain PRC193, was extensively investigated in various hybridization assays. The 350 Kb plasmid shows extensive relatedness with other fast-growing strains. This includes not only other plasmids which contain symbiotic genes, but also a fast-growing strain, PRC194, which has many sequences homologous to pRjaPRC193 in its chromosome. The endogenous plasmids in PRC194 show very little sequence relatedness to the probe pRjaPRC193. This indicates the fast-growing R. japonicum may have different DNA sequences represented in plasmids, but still retain the sequences of the symbiotic plasmid pRjaPRC193 in the chromosome or a plasmid. The plasmid pRjaPRC193 is moderately homologous to total DNA isolated from slow-growing strains. At least some of this

relatedness is due to conserved nif gene sequences. These hybridization results with pRjaPRC193 demonstrate the conservation of gene sequences, including symbiotic and other unidentified genes, between the fast-growing R. japonicum strains isolated from many diverse locations in the People's Republic of China. The fact such conservation exists suggests the sequences surrounding the known symbiosis genes may have direct and indirect roles in the symbiosis process.

The evidence shown for the conservation of plasmid sequences in the slow-growing strains indicated a strong amount of sequence conservation. Like the fast-growing R. japonicum strains examined, the slow-growing strains also were isolated from diverse geographical and ecological backgrounds. Although no genes associated with symbiosis and location on plasmid DNA have been determined in the slow-growing strains, it is nonetheless interesting that a high degree of plasmid DNA relatedness occurs between the strains. The plasmid DNA hybridizations between plasmids isolated from both slow- and fast-growing R. japonicum strains showed very limited homology. This suggests the two types of strains have distinct plasmid-borne DNA sequences.

Since so much information was being accumulated about the plasmid pRjaPRC193, from fast-growing R. japonicum strain PRC193, a physical and genetic map of the plasmid seemed an important task. The plasmid was digested with a variety of restriction enzymes to determine the

best choices for generating a physical map; the enzymes HindIII, HpaI, and KpnI were chosen. Size and repeated sequences were two problems encountered in the mapping of the large plasmid. The size problem was challenged by the use of cosmid cloning. Inserts up to 28 Kb were observed in a broad host range cosmid capable of replicating in both E. coli and R. japonicum. Single and double digests helped determine many of the overlapping cosmid clones for the assembly of the map in Figure 9.

The problem of repeated sequences greatly increased the difficulty of determining overlapping clones. There are several reasons for this. First, the common technique of 'chromosome walking', using a given clone as a hybridization probe, did not work well due to extra bands which hybridized. A certain amount of information was gathered, and in many occurrences the choices presented by the hybridization assays were used to fit HpaI or KpnI bands with the HindIII map. Suspected bands which might have fit with the HindIII map, and did not hybridize, provided valuable negative evidence. Another reason repeated sequences interfered with the mapping process is the over abundance of hybridization in the Southern cross restriction mapping procedure. Since over 15 sequences are repeated and distributed throughout the plasmid pRjaPRC193, as determined by the control HindIII cross, many hybridization signals developed and prevented extensive mapping of the plasmid in this manner. However, the Southern cross did provide convincing evidence of repeated

sequences, and furthermore, the size of each repeated sequence and the location of its copy was identified.

The third difficulty contributed by the repeated sequences is that one does not know which of several hybridizing bands represents a functional gene when a labeled gene probe hybridizes to homologous sequences in two or more map regions. In this study, two sets of nitrogen fixation and nodulation gene sequences were assigned to different locations on the map. Since the strain performs both symbiosis duties, at least one of each gene set must be functional, although it is not known which region contains the viable sequences. It is also possible both sets of genes are used by fast-growing R. japonicum. Therefore, genetic studies, perhaps involving transposon site-directed mutagenesis (Ruvkun and Ausubel, 1981), would have to be employed to solve this dilemma involving repeated sequences.

As much of a problem as the repeated sequences caused, their presence in the plasmid pRjaPRC193 did reveal a possible answer to an earlier question. Again, why is the plasmid in this strain, and other related strains, so large? At least one possible explanation is that recombination among similar sequences, perhaps involving insertion sequences, occurred over time in these strains. A certain amount of conservation, nitrogen fixation genes are a good example, must have been maintained under some selective pressure and perhaps balanced the amount of recombination events.

The bacterium Rhizobium japonicum is a complex, intriguing member

of the group of Rhizobium which enter symbiosis relationships with plant legumes. Many interesting facets of the special relationship R. japonicum has with the soybean plant will be revealed in the very near future. And true to its nature, the relationship will prove to be unique and unusual.

VI. LITERATURE CITED

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